

Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice

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Nerve growth factor (NGF) delivery to the brain of patients appears to be an emerging potential therapeutic approach to neurodegenerative disease, such as Alzheimer's disease (AD). The intranasal route of administration could provide an alternative to intracerebroventricular infusion and gene therapy. We previously showed that intranasal administration of NGF determined an amelioration of cholinergic deficit and a decrease in the number of phosphotau-positive neurons and of β -amyloid accumulation in AD11 mice, which express transgenic antibodies neutralizing NGF action and exhibit a progressive Alzheimer-like neurodegeneration. In this study, we report that the Alzheimer-like neurodegeneration in AD11 mice is linked to progressive behavioral deficits in visual recognition memory and spatial memory starting from 4 months of age. To establish whether intranasal administration of NGF, started after the appearance of the first memory deficits, could revert the cognitive deficits in AD11 mice, we assessed the performance of NGF-treated or control AD11 mice in the object recognition test and in a test of memory for place and context. Deficits exhibited by untreated AD11 mice could be rescued by the intranasal administration of NGF. Thus, this route of administration provides a promising way to deliver NGF to the brain in a therapeutic perspective.

Alzheimer's disease | behavior | mouse model

Nerve growth factor (NGF) (1, 2) is the most important target-derived trophic factor for basal forebrain cholinergic neurons (BFCNs). In rodents and nonhuman primates, NGF increases the synthesis of choline acetyltransferase and prevents BFCN atrophy caused by experimental injury or associated with physiological aging (3–8). Thus, NGF administration to the brain may counteract BFCN atrophy in physiological and pathological situations, such as aging and Alzheimer's disease (AD). This result, together with the fact that the progressive reduction of the BFCNs is responsible for the cognitive decline in AD patients, lays the groundwork to propose a therapeutic use of NGF in AD (9).

Despite the evidence of beneficial effects of NGF administration, therapeutic applications of NGF have several limitations. As for many other trophic factors, the blood–brain barrier represents a major problem in developing a NGF-based treatment for neurological diseases because it prevents this molecule from reaching the brain (10, 11). Until recently, the efficacy of NGF in rescuing BFCN atrophy was proved by using intracerebroventricular administrations to animal models (10, 12, 13). However, this route of administration is not practical in humans. A first clinical trial in AD patients, attempting to directly infuse NGF into human brain parenchyma, was suspended because of peripheral side effects of NGF (14). A second clinical trial, during which NGF was delivered by *ex vivo* gene therapy into the

brain with stereological injections, ameliorated cognitive deficits of AD patients (15). However, this gene therapy approach requires the use of risky surgical procedures to implant modified cells in the patients' brain parenchyma.

The development of a less invasive delivery method for NGF therefore may significantly improve the prospects of NGF clinical uses. Frey and coworkers (16, 17) showed that NGF and other trophic factors, such as IGF-I, can be delivered to the brain via the olfactory and/or trigeminal pathways. NGF can be transported to the rat brain via an extraneuronal route into the brain via intercellular clefts in the olfactory epithelium (18).

The demonstration that the intranasal NGF delivery was effective in rescuing neurodegeneration was achieved by using the AD11 anti-NGF mouse model for AD. AD11 mice express recombinant antibodies neutralizing NGF biological activity (19). As a result of NGF deprivation, AD11 mice show a progressive neurodegeneration characterized not only by atrophy of BFCNs and nucleus of Meynert, but also by the intracellular accumulation of phosphorylated insoluble tau and the deposition of β -amyloid (19–22). By using the intranasal route of administration, we showed that NGF could rescue, in a well defined time window, all of the histological hallmarks characterizing the AD-like neurodegeneration in AD11 mice (23).

AD11 mice exhibit clear spatial and visual recognition memory deficits (24, 25). Whether these deficits build up progressively, as suggested by the progression of the neurodegeneration (24–27) and synaptic plasticity impairment in the cortex (ref. 28 and N. Origlia, unpublished data) and hippocampus (E. Cherubini, unpublished data), is still unknown. Also unknown is whether intranasal NGF administration, started after the first memory deficits are already apparent, is able to counteract them.

In this study, the behavioral analysis of AD11 mice was extended to show the progression of the memory deficits, by using the object recognition test (ORT) to investigate visual recognition memory and the Morris water maze (MWM) to test spatial memory. We found that the first memory deficits are revealed by the ORT, in good accordance with the precocious appearance of hyperphosphorylated tau in the entorhinal cortex of AD11 mice. Under these experimental conditions, AD11 mice were tested to determine whether the intranasal route of administration of NGF could be used to revert recognition memory deficits. The results indicate that this hypothesis is indeed the case.

Abbreviations: NGF, nerve growth factor; BFCN, basal forebrain cholinergic neuron; AD, Alzheimer's disease; ORT, object recognition test; vORT, visual ORT; MWM, Morris water maze; OLT, object location test; OCT, object context test; DI, discrimination index.

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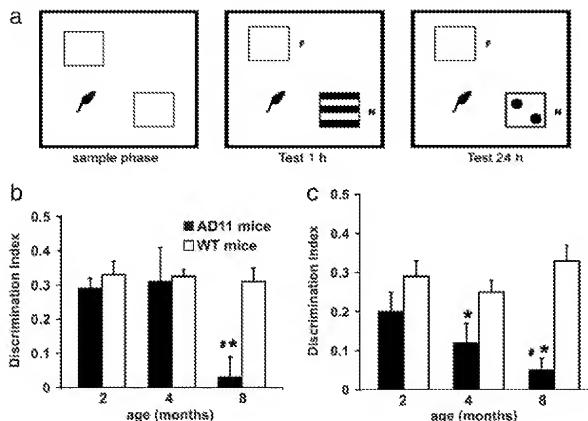


Fig. 1. Progression of vORT deficit in AD11 mice. (a) Schematic representation of sample and test conditions in vORT. (b and c) Performance in the vORT tested 1 h (b) and 24 h (c) after the end of the sample phase. The * denotes a significant ($P < 0.01$) difference between AD11 and WT mice at the given age (t test). The # denotes a significant difference with respect to the performance at 2 months of age for each genotype. Only AD11 mice show a significant decline of performance with age ($P = 0.009$ for the 1-h interval; $P = 0.02$ for the 24-h interval).

Materials and Methods

Visual ORT (vORT). The apparatus consisted of a square arena ($60 \times 60 \times 30$ cm) constructed in poly(vinyl chloride) with black walls and a white floor. The objects were cubes (12 cm wide) made of transparent Plexiglas that contained the visual patterns to be discriminated. The box and objects were cleaned between trials to stop the build-up of olfactory cues. Mice received three sessions of 10-min duration in the empty box to habituate them to the apparatus and test room. In the vORT, each mouse was first placed in the box and exposed to two identical sample stimuli (objects A1 and A2; e.g., two white cubes, 12 cm wide) for 5 min. This trial was called “sample phase” (Fig. 1a). The experimenter measured the total time the mouse spent exploring each of the two objects. Then the mouse was returned to its cage. During the 1- and 24-h retention interval, the experimenter removed both objects and replaced one of the two by its identical copy (A3) (to ensure that there was no carryover of olfactory cues) and the other object by a new one bearing a black-and-white pattern (object B). After a delay of 1 or 24 h, the mice were placed back in the box and exposed to the familiar object (A3, object identical to A1 and A2) and to a novel test object B for a further 5 min. The objects were placed in the same locations as the previous ones. The experimenter measured again the total time spent exploring each of the two objects (“test period”) (Fig. 1a). AD11 and age-matched wild-type (WT) mice were tested at 2 ($n = 9$ and 8, respectively), 4 ($n = 9$ for both), and 8 ($n = 10$ and 8, respectively) months of age.

MWM. To establish whether aged AD11 mice show deficits in object location, the MWM test was used. A circular water tank, made from black polypropylene (diameter, 100 cm; height, 40 cm) was filled to a depth of 25 cm with water (23°C) and rendered opaque by the addition of a small amount of milk powder. Four positions around the edge of the tank were arbitrarily designated north (N), south (S), east (E), and west (W), which provided four alternative start positions and also defined the division of the tank into four quadrants: NE, SE, SW, and NW. A circular clear Perspex escape platform (diameter, 10 cm; height, 2 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one of the four quadrants. Mice were trained for four

trials per day (with an intertrial interval of 30 min). The start position (N, S, E, or W) was pseudorandomized across trials. The hidden platform remained in the SW quadrant. Mice were allowed up to 60 sec to locate the escape platform, and their escape latency was recorded. On the last trial of the last training day, the mice received a single probe trial, during which the escape platform was removed from the tank, and the swimming path of each mouse was videorecorded over 60 sec while it searched for the missing platform. Mice at 4–5 (AD11 and WT mice, $n = 9$), 7 (AD11 and WT mice, $n = 9$), and 9 (AD11 mice, $n = 5$; WT mice, $n = 9$) months of age were tested in the MWM.

NGF Nasal Delivery. NGF administration was performed on anaesthetized mice as follows. First, 2,2,2-tribromethanol (Sigma-Aldrich) was dissolved in absolute ethanol at the concentration of 1 g/ml and stored at -20°C in the dark. After dilution in 0.9% NaCl at the final concentration of 2.5%, it was injected i.p. at the dosage of 10 $\mu\text{l/g}$ of body weight to induce anesthesia, which followed within 5–10 min after injection. After anesthesia, mice were laid on their back, with the head in upright position, as described in refs. 16 and 23. A 10- μM solution of mouse NGF (Alomone Labs, Jerusalem) in PBS was administered intranasally to AD11 mice, 3 μl at a time, alternating the nostrils, with a lapse of 2 min between each administration, for a total of 14 times. The administration was repeated seven times at 2-day intervals. During these procedures, the nostrils were always kept open. As control, AD11 mice were treated with PBS.

Rescue of Behavioral Deficits by NGF. This study was divided in two parts. In the first part, 42 AD11 mice were used for a standard vORT. In the second set of experiments, 35 AD11 mice were tested in a block of three experimental conditions as follows: (i) object (shape) recognition test (ORT); (ii) object location test (OLT); and (iii) object context test (OCT).

For the vORT habituation phase, NGF-treated and untreated AD11 mice were placed in the empty arena to become familiar with the apparatus for 5 min. The sample phase started after 2 min. Two cubes with white visual patterns were presented in two opposite corners of the arena. The mice were left to explore the

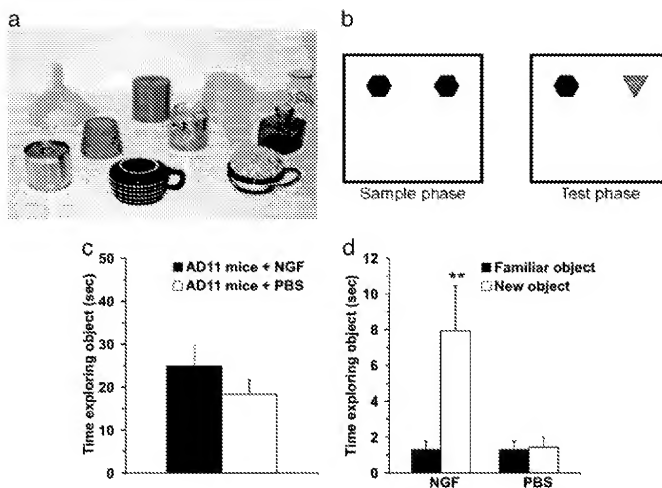


Fig. 2. NGF counteracts object-recognition deficits, as shown by the ORT. (a) Example of the objects used for the experiments. (b) Schematic representation of sample and test phase conditions in ORT. (c) Time spent in object exploration during the sample phase. There was no significant difference between the groups of treatment ($P = 0.487$). (d) Time spent exploring the familiar and the new objects in the test phase. The data show a significant difference within NGF-treated group ($P = 0.006$), whereas, for the PBS group, a significant difference was not found ($P = 0.813$). (Error bars represent SEM; **, $P < 0.01$.)

cubes in the arena for 5 min. The choice phase (5 min) was executed after 1 and 24 h.

The ORT consisted of two phases, sample and test. The objects to be discriminated were made of plastic, metal, and glass and were too heavy to be displaced by the mice (Fig. 2a). The objects varied in size; the largest was $\approx 6 \times 6 \times 10$ cm, and the smallest was $\approx 5 \times 5 \times 8$ cm. (Fig. 2a). During the sample phase, NGF-treated and untreated mice were placed into arena with two identical sample objects, allowed to explore for 5 min, and then returned to their cage for 10 min of retention interval. In this phase, the objects were placed in two adjacent corners of the arena (Fig. 2b). In the test phase, the objects were replaced with two new objects: one was identical to that used in the sample phase, whereas the other was a novel object that the mice had never encountered before (Fig. 2b). Mice were left to explore the objects for 3 min.

For OCT, two open field arenas ($60 \times 60 \times 30$ cm) made of poly(vinyl chloride) were used. Each arena constituted a different experimental condition (A and B). In condition A, horizontal white stripes were applied on the black walls of the arena. The floor was covered with rough Plexiglas (Fig. 3a). In condition B, the arena had gray walls, and the floor was made of Plexiglas (Fig. 3a). The particular object for a given test was randomly determined, but each object was used for only one experimental condition. Half of AD11 mice in each treatment group underwent the ORT in condition A, whereas the OLT was performed in condition B and vice versa. In this way, all of the mice were equally exposed to both environments before the OCT. The OCT was used to determine whether mice were sensitive to a change in context for a given object. Thus, previous familiarization with two environments was fundamental. The habituation phase started 2 days before the block of tests and consisted of four sessions. In each session, NGF-treated and untreated AD11 mice were exposed to both conditions (A and B). In the first and second sessions, mice were placed into the empty arena for 10 min. In the last two sessions, they were allowed to explore the

arena for 3 min individually. The OCT was divided into four sample phases and a test phase, each lasting 3 min (Fig. 3b). The retention interval within the sample phases was 2 min. There was a 5-min interval between the last sample phase and the test phase. In the sample phase, two objects were placed in adjacent corners of the arena; phases 1 and 4 comprised objects A₁ and A₂ in environment A, and phases 2 and 3 comprised objects B₁ and B₂ in environment B (Fig. 3b). The test phase was in the same environment as sample phase 4, but one of the objects (A₂) was replaced by B₂. In this way, one object was in the same environment as in the sample phase, and the other object was in a different environment from the sample phase (Fig. 3b). To avoid the eventual preference for one of two environments, half of the mice began the sample phase in environment A with object A₁ and A₂ and finished with the same environment with object A₁ and B₂ and vice versa.

The sample phase of the OLT was exactly the same as the ORT. After a delay of 10 min, the test phase began. In this phase, the objects were replaced by their identical copies, one of which was placed in the same position, whereas the other one was moved to the other adjacent corner, so that the two objects were now in diagonally opposite corners (Fig. 4a). Thus, in the test phase both objects were equally familiar, but one had changed location. The mice were exposed to the objects for 3 min.

Measurements and Statistics. The standard measure for the statistical analysis in the ORTs was the time spent exploring the two objects. The exploration of an object was defined as directing the nose to the object at a distance of < 2 cm and touching it with the nose. Turning around, climbing over, or sitting on the object were not included. In the sample phase, if the exploration time was < 3 sec, the mice were discarded from the sample. Mice also were excluded from the sample if they spent < 1 sec exploring both new and familiar objects in the test phase. In the sample phase, the total time spent exploring each object was recorded and compared across different genotypes or treatments with the

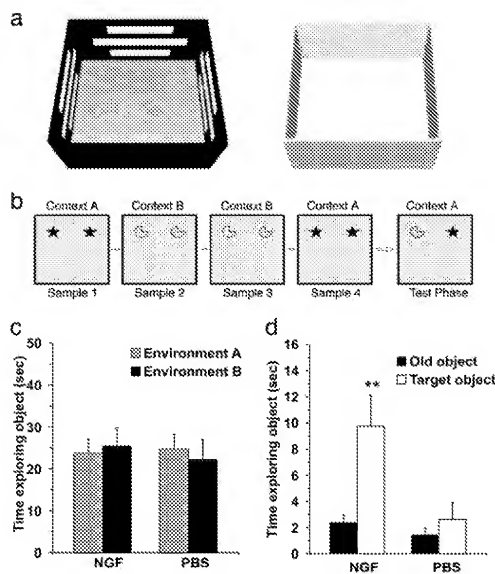


Fig. 3. NGF counteracts object-recognition deficits, as shown by the OCT. (a) Three-dimensional representation of conditions A (Left) and B (Right) in OCT. (b) Schematic representation of sample/test conditions in OCT. Condition A is represented by the shaded box, and condition B is shown by the plain box. Objects are represented by the symbols. (c) Object exploration time in the sample phase. There was no difference in both groups ($P = 0.600$). (d) Mean during exploration for the old object and the target object within each group. A clear difference was evident in the NGF-treated group ($P = 0.003$). For the PBS group, no significant difference was found ($P = 0.397$). (Error bars represent SEM; **, $P < 0.01$.)

Student *t* test and across different ages or different conditions with one-way ANOVA. For OCT, two-way ANOVA was used. In the test phase, comparisons between time spent exploring the new and old objects were performed within groups (analysis performed by using paired *t* tests). A discrimination index (DI) was calculated as the difference between the time spent exploring new and old object divided by the total time spent exploring the objects [$(n - f)/(f + n)$], where *n* represents new and *f* represents familiar. DIs were compared across ages for the same genotype with one-way ANOVA, across the two time intervals for the same genotype and age with a paired *t* test, and across genotypes or treatments for the same age and time interval with a *t* test. For the MWM, performance in the learning phase was compared with two-way ANOVA, time versus genotype, for repeated measures. Performance in the probe test was compared with one-way ANOVA across quadrants for each genotype.

Results

Progression of Behavioral Deficits in AD11 Mice. The vORT revealed that no differences in visual recognition memory between 2-month-old AD11 mice and age-matched WT mice could be shown. At this age, both groups spent significantly more time exploring the new object both at the 1- and the 24-h interval between the sample and the test phase. The DIs at 1 and 24 h did not differ between AD11 and WT mice. At 4 months of age, AD11 mice showed a deficit, with respect to WT mice (significantly lower DI, *t* test, $P < 0.05$), at the 24-h interval between

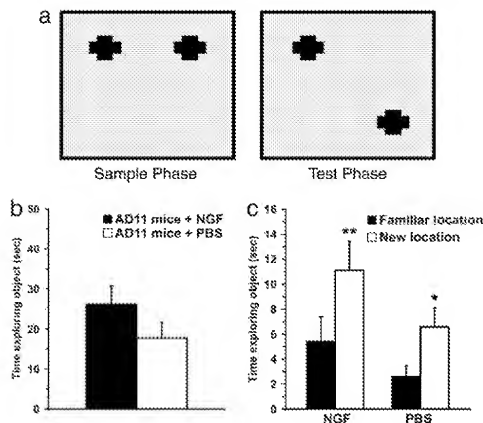


Fig. 4. NGF counteracts object-recognition deficits, as shown by OLT. (a) Representation of sample and test phase conditions in OLT. (b) Mean time spent exploring the objects during the sample phase. There was no significant difference between the groups ($P = 0.132$). (c) Mean time spent exploring the familiar and the new locations in the test phase. The data show a significant difference in both groups (NGF group, $P < 0.001$; PBS group, $P = 0.032$). (Error bars represent SEM; *, $P < 0.05$; **, $P < 0.01$.)

the sample and the test phase (Fig. 1b), whereas at 8 months of age, the deficits were observed both at 1- and 24-h interval (Fig. 1c, *t* test, $P = 0.006$ and $P < 0.001$, respectively). There was a clear decline of performance for AD11 mice with age for both 1- and 24-h intervals (one-way ANOVA, $P < 0.05$ and $P < 0.01$, respectively). On the contrary, the performance of WT mice did not significantly vary as a function of age (one-way ANOVA, $P > 0.05$).

We previously showed a progressive deficit in spatial memory tasks in an eight-arms radial maze paradigm (N.B., unpublished data). In the all-arms baited version of the radial maze, a deficit in the learning curves appeared starting from 4 months of age.

The MWM test showed a clear progression of spatial memory deficits. Five-month-old AD11 mice were able to learn the task as well as their age-matched WT controls, and the probe test was performed equally well (data not shown). At 7 months of age, AD11 mice showed significantly slower learning with respect to age-matched WT mice (two-way repeated-measures ANOVA: genotype, $P < 0.001$; days, $P < 0.001$, interaction, $P = 0.007$) (Fig. 5a). However, there was a significantly longer time spent in the target quadrant in the probe test for both genotypes (one-way ANOVA, $P < 0.001$) (Fig. 5b), indicating that AD11 mice remembered the location of the hidden platform at this age. At 9 months, AD11 mice showed a significantly worse performance in the learning curve (two-way ANOVA, genotype, $P < 0.01$) (Fig. 5c) and did not remember the location of the hidden platform in the probe test (Fig. 5d; $P > 0.05$).

Rescue of Behavioral Deficits by NGF. vORT. Fig. 6a shows the time spent exploring objects in the sample phase. There were no group differences in exploration time (Student's *t* test, $P = 0.481$). These data indicated that, if any significant differences between the groups in terms of discrimination on the test phase were observed, they did not result from differences in time spent exploring in the sample phase. In the test phase, NGF-treated AD11 mice spent significantly more time with the new object after the 1-h (Fig. 6b) and 24-h (Fig. 6c) delay (paired *t* test, $P =$

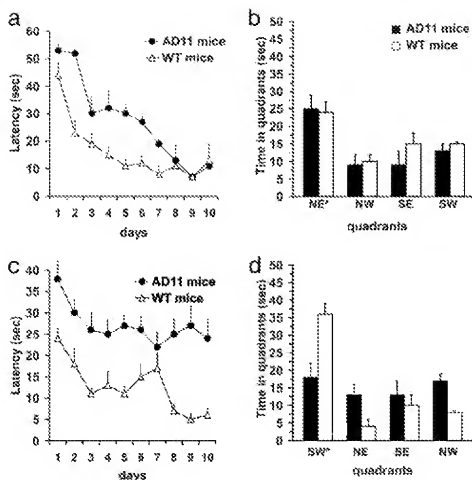


Fig. 5. Progression of MWM deficit in AD11 mice. (a) Learning curves for WT and AD11 mice at the age of 7 months. The difference between WT and AD11 mice is significant (genotype, $P < 0.001$; time, $P < 0.001$). (b) Results of the probe trial at 7 months of age. The platform (located in the NE quadrant during learning) was removed. Both WT and AD11 mice spent significantly more time in the NE quadrant ($P < 0.001$). (c) Learning curves for AD11 and WT mice ages 9–10 months. The difference between WT and AD11 is significant (genotype, $P < 0.01$; time, $P < 0.01$). (d) Results of the probe trial at 9–10 months of age. The platform (located in the SW quadrant during learning) was removed. WT mice spent significantly more time in the SW quadrant, whereas AD11 mice did not show any significant preference for the SW quadrant ($P < 0.001$ and $P > 0.05$, respectively).

0.001 and $P = 0.005$, respectively). The placebo group did not show a significant difference in either 1- or 24-h delay (paired t test, $P = 0.282$ and $P = 0.138$, respectively). In the test phase (Fig. 6d), the DI data revealed that the NGF-treated group had an exploration index that was significantly higher than the PBS-treated group in 1-h delay (ANOVA, $F = 8.896$; $P = 0.007$), whereas in the 24-h delay there was not a significant difference between the two groups of animals (ANOVA, $F = 1.132$; $P = 0.300$).

Object (shape) recognition test. During the sample phase, analysis of the total time spent in exploration revealed no significant differences between the NGF- and PBS-treated groups (Student's t test, $P = 0.487$) (Fig. 2c). The NGF-treated group of AD11 mice showed at the 10-min time interval a significantly greater exploration time dedicated to the novel object compared with the familiar object (paired t test, $P = 0.006$), whereas the PBS-treated group did not show a significant difference (paired t test, $P = 0.813$) (Fig. 2d). These data confirmed that AD11 mice treated with PBS failed to discriminate the novel object, whereas the NGF-treated AD11 mice clearly were able to discriminate between the two objects. One-way ANOVA revealed an effect of NGF treatment ($F = 6.215$; $P = 0.025$) indicated in the fact that the NGF-treated group of mice had a DI significantly higher than those of PBS group (Fig. 7).

OCT. In the sample phase of context version, taking into account both environments, two-way ANOVA (treatment vs. environment) showed that there was not a significant difference for either factor ($P = 0.783$ for treatment factor; $P = 0.914$ for the environment factor). The effect of different treatments did not

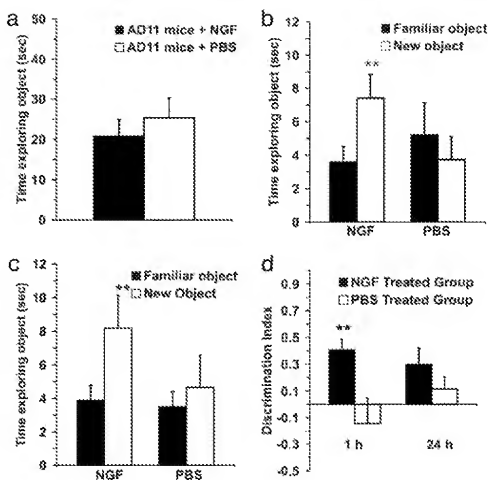


Fig. 6. NGF counteracts object recognition deficits, as shown by the vORT. (a) Time engaged in object exploration during the sample phase for vORT. There was not a significant difference between the groups. (Error bars represent SEM.) (b) Time engaged in exploring each object type (new and familiar) during the test phase performed 1 h after the end of the sample phase. (Error bars represent SEM; **, $P < 0.01$.) (c) Time engaged in exploring each object type (new and familiar) during the test phase performed 24 h after the end of the sample phase. (Error bars represent SEM; **, $P < 0.01$.) (d) DI in the vORT. Data revealed that in the 1-h delay there was significant difference between the groups ($P = 0.007$), whereas in the 24-h delay there was not a significant difference ($P = 0.300$). (Error bars represent SEM; **, $P < 0.01$.)

depend on what environment was present. There was no statistically significant interaction between both factors ($P = 0.600$). These data showed no significant preference between the two environments (Fig. 3c). In the test phase, one paired t test was used to verify the eventual difference in the exploration time between the object that was in the changed environment (target object) and the object that was in the same environment (old object). The results showed that the NGF-treated group was able to discriminate between the old and target objects ($P = 0.003$), whereas no significant difference was found in the PBS group ($P = 0.397$) (Fig. 3d). One-way ANOVA indicated that the

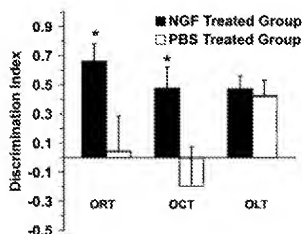


Fig. 7. DI in each experimental condition. The graph shows that the performance of the NGF-treated group was greater than that of the PBS group in the ORT and OCT ($P = 0.025$ and $P = 0.045$, respectively). OLT revealed that there was not a significant difference between treated and untreated mice ($P = 0.760$). (Error bars represent SEM; *, $P < 0.05$.)

NGF-treated group could discriminate between the two objects significantly better than the PBS group ($F = 4.68$ and $P = 0.045$) (Fig. 7).

OLT. The analysis in the sample test revealed that no significant differences were found in the total amount of exploration time between the two groups of mice (Student's t test, $P = 0.132$; Fig. 4b). In the test phase, exploration times for both groups of treatment demonstrated a clear preference of the object placed in a novel location compared with the object placed in a familiar location (paired t test: NGF-treated group, $P = <0.001$; PBS group, $P = 0.032$) (Fig. 4c). The PBS-treated AD11 mice showed they were able to discriminate the novel location from the familiar location. In fact, one-way ANOVA between the groups, considering the DI, revealed that there was no significant difference between groups of treatment ($F = 0.096$ and $P = 0.760$) (Fig. 7).

Discussion

The main findings of this study were that AD11 mice showed a progressive behavioral deficit and that NGF intranasal delivery increased the ability of AD11 mice in remembering a familiar object and in associating an object to a particular context.

We used tasks that exploited the rodents' spontaneous preference for novel objects, which were first introduced by Ennaceur and Delacour (29). The introduction of these tests allowed overcoming the disadvantages of lengthy training procedures. More importantly, the ORT strongly relies on visual memory. Visual recognition memory is currently under examination as a potential early diagnostic marker of AD, because neurofibrillary tangles initially develop in subregions of the parahippocampal gyrus known to be important for visual recognition memory (24, 25, 30, 31). In this context, it is very interesting that in AD11 mice, the first memory deficits become apparent during the vORT, not in the MWM, in line with the first appearance of hyperphosphorylated tau in the entorhinal cortex (26). ORTs have the advantage that, in addition to examining the exploration of a novel object, they can be used to examine other aspects of recognition, such as object location and context. Thus, both

spatial and nonspatial working memory can be tested by using the same paradigm (22), allowing a more stringent comparison between deficits in spatial and nonspatial memory.

In this study, we show that AD11 mice display progressive behavioral deficits in visual recognition and spatial memory. Although impairment in object recognition starts at 4 months of age, deficits in spatial memory appear later (9 months of age), as assessed by using the MWM. In keeping with these latter findings, the OLT performed at 6 months of age did not reveal any impairment in AD11 mice. In addition, AD11 mice were found to be insensitive to the combination of object and environment. Thus, unlike normal rats and mice (32), but as in rats with hippocampal damage (27), AD11 mice cannot link the event of exploring and experiencing an object with the contextual cues surrounding that particular object, not being able to provide parallels with episodic memory. The deficits in object and contextual recognition both were counteracted by the intranasal administration of NGF.

In conclusion, although it was already shown that the intranasal administration of trophic factors, such as insulin, can improve memory and mood in healthy (33) and AD subjects (M. Roger and S. Craft, unpublished data), there was no evidence for such an effect after NGF intranasal delivery. Thus, to our knowledge, this study provides, for the first time, the evidence that the noninvasive intranasal administration of NGF determines not only the rescue of the main hallmarks of AD-like neurodegeneration, such as phosphotau and β -amyloid (23), but also counteracts functional cognitive deficits. In the context of the emerging role that NGF and its precursors could play in the onset and therapy of AD, these results highlight the possibility that the olfactory pathway can be a promising, noninvasive route of administration for the delivery of NGF agonists, allowing a long-term treatment of AD.

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A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease

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Cholinergic neuron loss is a cardinal feature of Alzheimer disease. Nerve growth factor (NGF) stimulates cholinergic function, improves memory and prevents cholinergic degeneration in animal models of injury, amyloid overexpression and aging. We performed a phase 1 trial of *ex vivo* NGF gene delivery in eight individuals with mild Alzheimer disease, implanting autologous fibroblasts genetically modified to express human NGF into the forebrain. After mean follow-up of 22 months in six subjects, no long-term adverse effects of NGF occurred. Evaluation of the Mini-Mental Status Examination and Alzheimer Disease Assessment Scale-Cognitive subcomponent suggested improvement in the rate of cognitive decline. Serial PET scans showed significant ($P < 0.05$) increases in cortical 18-fluorodeoxyglucose after treatment. Brain autopsy from one subject suggested robust growth responses to NGF. Additional clinical trials of NGF for Alzheimer disease are warranted.

Inexorable loss of synapses and neurons occurs in Alzheimer disease over time, leading to cognitive decline. Growth factors prevent neuronal death and improve memory in animal models of neuron death, excitotoxicity, aging and amyloid toxicity^{1–7}, suggesting that they may be useful for treating disease resulting from neuronal degeneration, including Alzheimer disease.

The first discovered growth factor, NGF⁸, specifically targets basal forebrain cholinergic neurons, nociceptive dorsal root ganglion neurons and some third-order sympathetic neurons. The former neuronal group is of particular importance in Alzheimer disease⁹, is most common neurodegenerative disorder. Basal forebrain cholinergic neurons release the majority of acetylcholine in the cerebral cortex and hippocampus¹⁰, enhancing synaptic efficacy and modulating active cortical circuits^{11,12}. These cholinergic neurons profoundly degenerate in Alzheimer disease¹³, contributing to cognitive decline.

That NGF might reduce cholinergic cell loss in Alzheimer disease has been appreciated, for some time, however, delivering NGF to the

brain in a safe manner is challenging. NGF does not cross the blood-brain barrier when administered peripherally because of its size and polarity. Yet when infused into the brain ventricular system, NGF causes intolerable side effects from its broad distribution, including pain (from stimulating dorsal root ganglion nociceptive neurons), weight loss (from hypothalamic stimulation) and Schwann cell migration into the spinal cord and meninges¹⁴. For clinical use, NGF administration must therefore meet two requirements: it must be delivered in sufficient quantities to effectively stimulate neurons, and its distribution must be restricted to sites of degenerating neurons to avoid adverse effects. Gene delivery meets these requirements. Using either *ex vivo* (genetic modification of cells *in vitro*) or *in vivo* (genetic modification of cells in the brain itself) gene therapy, growth factors can be delivered directly to the brain and diffuse for distances of 2–5 mm². This clinical trial was undertaken based on extensive preclinical studies, including primate studies, showing safety and efficacy of *ex vivo* NGF gene delivery in preventing cholinergic basal forebrain neuronal death and stimulating cell function^{15,16,18}.

We enrolled eight subjects (five female, three male; mean age 67.2 ± 2.6 years, range 54–76 years) with early-stage probable Alzheimer disease in our study. NGF was administered to the brain using *ex vivo* gene delivery. Animal studies indicate that primary autologous fibroblasts genetically modified to produce and secrete human NGF survive grafting to the brain and sustain NGF production for at least 18 months, prevent cholinergic degeneration, stimulate cholinergic function and improve memory^{14–18}. Further, implanted cells do not form tumors, do not migrate and cause no discernable toxicities in dose-escalation studies¹⁷. Implementing this procedure in humans, autologous fibroblasts obtained from small skin biopsies in each subject were genetically modified to produce and secrete human NGF using retroviral vectors¹⁹. NGF production was measured, and cells were stereotactically injected into the cholinergic basal forebrain (a region ~1 cm in length) in one surgical session. The first two subjects received injections into only the right brain; the following six subjects received bilateral injection.

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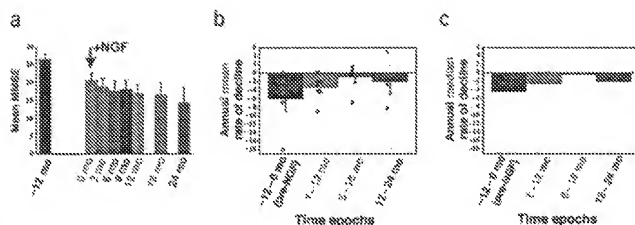


Figure 1 Cognitive outcome, MMSE. (a) Mean total MMSE scores at time of screening, 1 year later (2 weeks before NGF treatment), and at subsequent intervals. Error bars, s.e.m. (b) Mean annualized change in MMSE score in year before treatment, and in time epochs of 1–12 months, 6–18 months and 12–24 months after treatment. Individual subject data points shown in circles. Of note, during 1-year period beginning 6 months after treatment, when sufficient time passed for NGF to enhance cholinergic projections, two of six subjects showed improved MMSE scores, one subject had no decline, and two subjects declined only 1 point. Overall decline was reduced 51% compared to preoperative rate for the mean 22-month period after treatment. (c) Given wide range in MMSE scores, median data also are shown and parallel observations of mean scores.

tions. Cognitive outcomes were measured at regular intervals. Serial PET scans were obtained 6–8 months apart in four bilaterally treated subjects using 18-fluorodeoxyglucose (FDG) imaging; two subjects underwent preoperative and 6-month postoperative scans, and two subjects had two serial postoperative scans at 10 and 18 months after delivery of NGF.

Stereotaxic injections were completed safely in six of eight subjects. The trial began with injections performed while subjects were awake but sedated. Two subjects abruptly moved while the injection needle was in the brain, causing subcortical hemorrhage. All subsequent procedures were performed under general anesthesia to avoid unanticipated movement, and were completed safely. Neither hemorrhage required surgical evacuation. The hemorrhage in the first subject initially caused stupor with left hemiparesis. This subject improved slightly over time, but sustained pulmonary embolism and cardiac arrest, and died 5 weeks after the surgery. Hemorrhage in the second subject caused right hemiparesis and exacerbation of dementia-associated aphasia; hemiparesis recovered and aphasia improved modestly.

We detected no adverse effects attributable to delivery of NGF itself or to the gene-delivery vector after 18–24 months of monitoring, including weight loss or pain. Subject 7 experienced two syncopal episodes 5 weeks and 1 year after gene delivery, possibly related to the procedure. All other adverse events (one episode each) included knee effusion, wrist fracture, gout and intermittent moderate low back pain (history of this pain preceded NGF and did not change after treatment).

Cognition was assessed in six subjects solely completing NGF delivery. Mean Mini-Mental Status Examination (MMSE) scores before treatment showed a mean annual rate of decline of 6.1 ± 2.7 points per year, based on pre-treatment assessment over 14.6 ± 2.6 months (range 10–27 months, Fig. 1). Postoperatively, over an average period of 22 months, the mean rate of MMSE decline was 3.0 ± 1.0 points per year, a reduction of 51% compared to the preoperative rate. Notably, in preclinical studies in aged primates¹⁸, NGF gene delivery to the basal forebrain requires several months to enhance cortical cholinergic terminal density;

such enhancement probably underlies beneficial effects on memory^{2,19}. Enhancement of cortical cholinergic terminals would also require several months in the larger human brain; thus, we examined time epochs after NGF delivery (Fig. 1). During the period 6–18 months after treatment, when NGF expression remained robust and sufficient time has passed to strengthen cortical cholinergic terminals¹⁸, two of six subjects showed improved MMSE scores, one showed no decline and two showed only a minor decline of 1 point per year; the remaining subject declined 7 points. Compared to the time before treatment, MMSE decline during the 6–18 month interval after surgery was significantly reduced by 84% ($P < 0.05$, paired *t*-test). Because MMSE scores varied widely in this small sample, median decline rates are also shown (Fig. 1) and are consistent with mean values.

Cognition was measured on a second scale commonly used in Alzheimer disease trials, the Alzheimer Disease Assessment Scale-Cognitive subcomponent (ADAS-Cog). Preoperative rate-of-decline data on this scale were not collected; published rates of decline in individuals with moderately severe Alzheimer disease range from 3–10 points/year, with an approximate average of 6 points/year^{20,21}. After treatment, mean annual decline on the ADAS-Cog over a 22-month period was 6.2 ± 1.9 points (Fig. 2). Analysis of time epochs of 1–12, 6–18 and 12–24 months paralleled findings on the MMSE scale, with a 56% improvement in rate of decline comparing 12–24 months after NGF treatment to 1–12 months after treatment. Analysis of median decline rates showed amelioration of decline by 55% at 6–18 months compared to 1–12 months after treatment.

PET scans in four bilaterally treated subjects showed significant increases in FDG uptake on the second scan compared to the first ($P < 0.05$), a reversal of usual decline in Alzheimer disease²² (Fig. 3). Increases were observed in most cortical regions receiving cholinergic input from nucleus basalis. In contrast, the striatum, which does not receive projections from the nucleus basalis, showed no change in FDG. Increased FDG uptake was also evident in the cerebellum, a structure showing plasticity associated with cognition and attention^{23,24} and bearing direct and indirect reciprocal projections to cortex. Subjects were not receiving cholinesterase inhibitors or memantine.

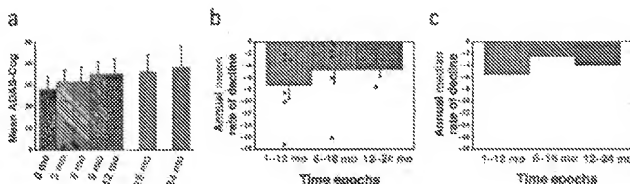


Figure 2 Cognitive outcome, ADAS-Cog. (a) Mean total ADAS-Cog scores 2 weeks before NGF and subsequently. Error bars, s.e.m. (b) Mean annualized change in ADAS-Cog over time epochs of 1–12 months, 6–18 months and 12–24 months after treatment. Individual subject data points shown in circles. As with MMSE, rate of decline slows after sufficient time has passed (6 mo) for NGF to enhance cholinergic systems¹⁸. Rate of decline is reduced by 56% at 6–18 months compared to 1–12 months. (c) Given wide range in ADAS-Cog, median data also are shown. Rate of decline is slowed by 55% at 6–18 months compared to 1–12 months.

With consent, we examined the brain of the subject who died 5 weeks after NGF delivery. A diagnosis of Alzheimer disease with Lewy body disease was confirmed, with numerous amyloid plaques (Braak stage 4), neurofibrillary tangles and Lewy bodies in the brainstem, substantia nigra and cortex. Nissl-stained forebrain sections showed survival of implanted autologous fibroblasts with typical differentiated, spindle-shaped morphology (Fig. 4) observed in monkey studies.¹⁷ Occasional granulomatous cells were present, as observed in monkey studies, but other inflammation was not detected. *In situ* hybridization for messenger RNA encoding NGF showed robust NGF expression in grafts, and immunocytochemistry showed robust sprouting of cholinergic axons into NGF delivery sites (Fig. 4).

Gene therapy meets the goals of site-directed growth factor delivery. Now reporting the first testing of NGF gene delivery to humans, we found that the approach had no long-term adverse effects. But subjects must be anesthetized to allow safe completion of the stereotaxic delivery.¹⁵ We also found three potential beneficial effects of NGF gene delivery. First, NGF induced 'trophic' responses, with robust cholinergic axon sprouting into the site of NGF delivery. This constitutes the first demonstration of the sensitivity of the human brain to growth factors, and the extent of the sprouting is similar in magnitude to that seen in young and aged monkeys receiving NGF.^{18–19} Sensitivity to NGF is thus retained by degenerating neurons in Alzheimer disease. Second, PET scans showed broad increases in glucose uptake by cortical neurons after NGF delivery in humans. A previous, short intraventricular infusion trial of NGF protein in three individuals with Alzheimer disease reported improvements in cortical nicotine receptor activation by PET.²⁶ The extensive distribution of these increases in our study mirrors cortical patterns of innervation by cholinergic projections arising from nucleus basalis¹⁰, suggesting that cholinergic projections to cortex are activated by growth factors in Alzheimer disease. Third, with the caveat that this is a small, non-placebo-controlled patient cohort undergoing open-label treatment and unblinded analysis, we found that the rate of disease progression seemed to be reduced by 36–51% for a mean period of nearly 2 years on two common clinical measures of cognitive function in Alzheimer disease. In the 6–18-month period after NGF delivery, corresponding to the predicted period of active growth factor enhancement of cholinergic cortical projections¹⁰, cognition apparently improved or stabilized in five of six subjects. By comparison, currently approved medications for Alzheimer disease have an estimated impact on these cognitive measures of 5%²⁷, and are not known to affect decline over prolonged periods.

NGF was delivered to the cholinergic nucleus basalis in our study, a subcortical region of modest size yet with extensive projections throughout the cortex. This broad cholinergic projection facilitates cortical plasticity and maintains neuronal activation^{1,12}, actions with broad implications for cortical function. Indeed, declines in cholinergic systems probably contribute to the extent and diversity of cortical dysfunction in Alzheimer disease^{13,28,29}, although the magnitude of cholinergic contribution to cognitive failure in Alzheimer disease has not been clear. Cholinergic neurons are just one of several cell systems degenerating in Alzheimer disease. Because the actions of NGF are restricted to cholinergic systems in the forebrain, NGF delivery provides an opportunity to examine whether potent enhancement of just the cholinergic component of neuronal degeneration in Alzheimer disease can substantially impact broader cortical function and maintain effects over time. Our PET findings indicate that NGF stimulation indeed broadly increases cortical glucose uptake. The possible amelioration of cognitive decline also supports a broader impact of growth factor stimulation of cholinergic neurons in Alzheimer disease, although definitive statements require placebo-controlled, blinded trials. Should properly

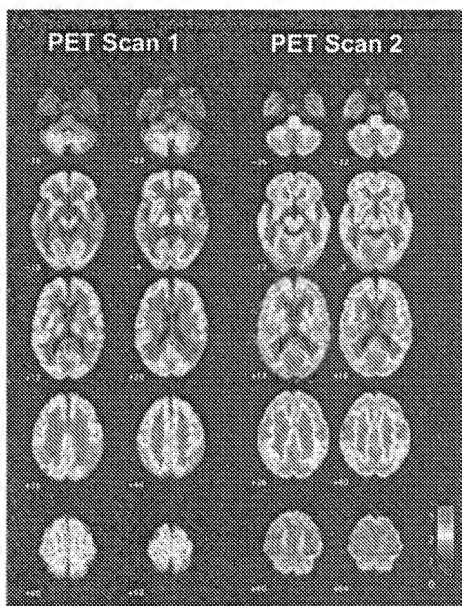


Figure 3 PET scans. Averaged FDG PET scans in four subjects treated with NGF, overlaid on standardized MRI templates. Representative axial sections, with 6–8 months between first and second scan, showing widespread interval increases in brain metabolism. Flame scale indicates FDG use/100 g tissue/min; red color indicates more FDG use than blue.

controlled future trials confirm that NGF gene delivery reduces cognitive decline to the extent and for the duration we observed, it would be an important advance over current treatments for Alzheimer disease. But NGF is unlikely to 'cure' the disease. Alzheimer disease pathology includes amyloid accumulation, neurofibrillary degeneration, synapse loss and cell loss, and it is untenable that therapy directed solely at cholinergic neurons will interrupt these broad pathogenic mechanisms. Nonetheless, slowing of clinical decline to the degree observed in this trial over periods of even a few years would represent a useful therapy substantially surpassing the efficacy of current treatments for Alzheimer disease. Further, NGF could act synergistically with antiamyloidogenic approaches³⁰ to provide greater efficacy than either treatment alone.

The findings of improvement in PET activity and a possible effect on clinical outcome mirror recent findings after delivery of a dopaminergic growth factor, glial cell line-derived neurotrophic factor (GDNF), in Parkinson disease³¹. In the Parkinson disease trial, growth factors were restricted to specific striatal targets by continuous intraparenchymal GDNF protein infusions. Subjects showed increases in fluorodopa uptake at infusion sites and improvement in clinical rating scales. Whereas primary endpoints were not achieved in a phase 2 GDNF infusion trial in Parkinson disease, only half the dose of the earlier trial was used. Another phase 1 GDNF intraparenchymal infusion trial recently reported significant ($P < 0.05$) clinical improvement using higher GDNF doses and an infusion technique widening protein delivery³². Together with our findings, there is early but suggestive evidence

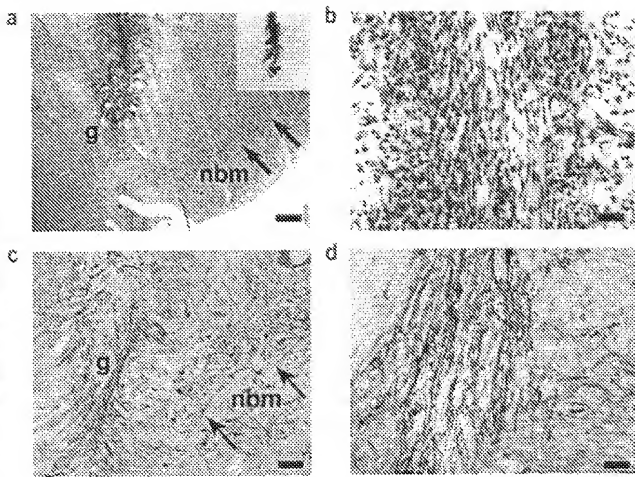


Figure 4 Tropic response to NGF in the human brain. (a,b) Nissl stain of autologous, NGF-secreting cell implant in brain of individual with Alzheimer disease 5 weeks after treatment. Graft (g) adjacent to nucleus basalis of Meynert (nbm, arrows). Inset, robust mRNA encoding hGF by *in situ* hybridization within graft. Scale bar in a, 247 μ m; in b, 24 μ m. Note proximity of graft to nbm, seen in similar perspective in c at higher magnification. (c,d) Immunocytochemistry for cholinergic neurons ($\alpha 7$). (c) shows graft implant on left (g) and adjacent neurons of nbm (arrows). Higher magnification (d) shows dense penetration of cholinergic axons into graft. Scale bar in c, 82 μ m; in d, 11 μ m.

that, when administered into the central nervous system in therapeutic doses and a regionally restricted manner, long-term growth factor treatment is well tolerated and has the potential to improve symptoms and modify neurodegenerative disease progression.

METHODS

Subjects. We enrolled eight subjects, with a mean duration of Alzheimer disease diagnosis of 2.1 ± 0.2 years, and mean time from first symptom onset of 4.6 ± 0.7 years (\pm s.e.m.). The study was approved by the University of California San Diego Human Research Protection Program and the US Food and Drug Administration, reviewed by the US National Institute of Health Recombinant Advisory Committee, and monitored by a data-safety monitoring board. Informed consent was obtained and history, physical, neurological examination, brain imaging (review of magnetic resonance imaging or computed tomography scan within last year) and MMSE were performed. Inclusion criteria were as follows: (i) diagnosis of probable Alzheimer disease (criteria of NINCDS-ADRDA workgroup³⁵), (ii) age 50–80 years, (iii) MMSE score 20–28 and (iv) absence of exclusionary factors including active medical or psychiatric problems, surgical contraindications or inability to understand and repeat purpose and risks of the study. Anticholinesterase therapy was discontinued 3 months before hGF. Excluded medications included neuroleptics and sedatives (memantine was unavailable at study onset). Stable doses of baseline medications were maintained. Consent was provided by subject and caregiver.

To obtain primary autologous fibroblasts genetically modified to produce and secrete full-length, human β -NGF, 3 \times 3 mm skin biopsies from the back were dissociated *in vitro* and transduced using modified Moloney leukemia virus vectors³⁶ or Molecular Medicine, Inc. Transduced cells were selected with antibiotics added to culture media. NGF production, measured by enzyme-linked immunosorbent assay, ranged between $25\text{--}75$ ng NGF/ 10^6 cells/d. Production of 'pro-NGF' (a variant of 'mature' NGF) was measured because of reported association with cell loss³⁷, but constituted less than 1% of mature NGF (Supplementary Fig. 1 online). Thus, secreted NGF was nearly entirely of cell survival-promoting form.

Surgery. Subjects underwent preoperative stereotaxic MRI to localize the nucleus basalis of Meynert (NBM). Five sites, equally spaced over the rostral-to-caudal extent of NBM on each side of the brain were identified and injected with genetically modified autologous cells through a single burr hole on either frontal cranium, using a Leksell stereotaxic apparatus, at a rate of 1 μ l/min. The needle remained in place 5 min before withdrawal. Subjects 1 and 2 received injections into only the right NBM, 500,000 cells per site (2.5×10^6 total cells/concentration, 100,000 cells/ μ l; 5 μ l injected into each of five sites). Subjects 2–6 received 500,000 cells per site into both right and left NBM (5.0×10^6 total cells/concentration, 100,000 cells/ μ l; 5 μ l injected into each of five sites per side, ten sites total). Subjects 7–8 received 1,000,000 cells per site into both right and left NBM (10.0×10^6 total cells/concentration, 100,000 cells/ μ l; 10 μ l injected into each of five sites per side, 10 sites total). Treatment of different dose cohorts was staggered by 3 months, allowing extended observation.

Testing. We measured screening and baseline MMSE. At study initiation, comparison of pre- to postoperative rate of decline on the MMSE was not planned; however, after study initiation it became evident that the pre-treatment observation period was of sufficient duration (14.6 months) to allow such an analysis, and the intent to compare preoperative to postoperative rate of decline was therefore adopted after treating subject 3. MMSE and ADAS-Cog³⁸ were measured 2 weeks preoperatively and at 3-month intervals postoperatively for 1 year, at 18 months, and in some cases, 24 months after treatment. Comparison of preoperative (from screening to baseline assessment) and postoperative decline rates on the MMSE were made using paired *t*-tests, with a significance level of $P < 0.05$ (Statview software). Concomitant medications, adverse events, physical and neurological examination, weight, back pain questionnaires and serum chemistries were checked each visit. Magnetic resonance scans were performed at 6, 12 and 24 months to screen for brainstem cell hyperplasia⁷. PET scans were obtained at the University of California Irvine Brain Imaging Center in four subjects safely injected bilaterally, using a GE 2048-15B scanner, as previously described³⁷. Subjects named 260 objects pictured in the Snodgrass and Vanderwart set immediately before receiving intravenous injection of FDG (~ 5 μ Ci). We obtained 30 slices at 5.5-mm intervals, with attenuation correction by 68Ga pin source transmission scan.

Histopathology. Following consent, we removed the brain within 5 h after death, sectioned it into blocks, and immersion fixed it in 4% paraformaldehyde for 72 h. We performed thionin staining, immunolabeling for cholinergic cell markers (choline acetyltransferase and p75 receptor), and *in situ* hybridization for mRNA encoding NGF on sections that were 40 μ m thick³⁷.

Statistical analyses. Postoperative rates of cognitive decline were compared to pre-operative rates of decline using student paired *t*-test. PET statistics, before statistical analysis, images were scaled to canonical normal cerebral blood flow of 30 ml/min/dl to account for global blood flow changes over time. Regionally specific condition effects were compared using linear contrasts; the resulting voxel values represented a parametric mapping of *t*-statistics, SPM (*t*), which were transformed into the unit normal distribution SPM (*z*) and thresholded at $t = 2.12$, $P < 0.05$. Resulting foci were characterized in peak height (*g*). The significance of each region was estimated based on the probability that the observed peak height could have occurred by chance ($P_{\text{peak}} > \alpha$). Spatial extent thresholding based on Gaussian random field theory was performed at 10 voxel, to correct for multiple comparisons.

See: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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Research report

Continuous intrathecal fluid infusions elevate nerve growth factor levels and prevent functional deficits after spinal cord ischemia

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Abstract

Continuous intracerebroventricular or intrathecal infusions of neurotrophic factors have been reported to prevent neuronal degeneration, stimulate axonal sprouting and ameliorate behavioral deficits in various models of CNS injury and aging. In the present study, the ability of intrathecal infusions of recombinant human nerve growth factor (NGF) to reduce functional deficits following spinal cord ischemia was investigated. Adult rabbits underwent intrathecal cannulation and continuous infusions of either 300 µg/ml recombinant human NGF or artificial CSF (vehicle) at a rate of 143 µl/day for 7 days prior to induction of spinal cord ischemia. Continuous infusions were maintained after induction of ischemia. Four days later, both NGF-treated and vehicle-infused subjects showed a significant amelioration of functional motor deficits compared to lesioned, non-infused subjects ($P < 0.05$). The average duration of tolerated ischemia increased from 23.4 ± 1.8 min in lesioned, non-infused subjects to 35.5 ± 3.1 min in lesioned, artificial CSF-infused subjects and 35.6 ± 4.7 min in NGF-infused subjects (mean \pm S.E.M.). Significantly elevated NGF protein levels were attained within the spinal cords of both NGF-treated subjects and artificial CSF-infused subjects, although levels were substantially higher in NGF-treated subjects (9.8 ± 3.8 ng/g in NGF-infused vs. 2.0 ± 0.4 ng/g in vehicle-infused and only 0.4 ± 0.2 ng/g in lesioned, non-infused animals). These findings indicate that the process of intrathecal cannulation and fluid infusion elicits alterations in the spinal cord environment that are neuroprotective, including spontaneous elevations in NGF levels. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration**Topic:** Neurotropic factors: biological effects**Keywords:** Nerve growth factor; Ischemia; Neurotrophin; Spinal cord; Functional recovery

1. Introduction

Neurotrophic factors prevent neuronal degeneration and promote axonal growth in responsive cell populations in various regions of the developing and adult mammalian nervous system [7]. Neurotrophic factors such as nerve growth factor (NGF) help to maintain normal neuronal function and also sustain the phenotype of neurons following injury to the nervous system. Neurotrophin levels become elevated in some regions of the nervous system after trauma, as a result of either increased production or diminished utilization by responsive neurons [22]. For example, Schwann cells in injured peripheral nerves

upregulate expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) mRNA and increase NGF protein production [18]. NGF levels rise in the hippocampus following axotomy of cholinergic inputs, presumably as a result of reduced uptake and retrograde transport by cholinergic terminal [15]. NGF administration prevents degeneration of septal cholinergic neurons after axotomy [13], and implantation into the brain of fibroblasts that are genetically modified to secrete NGF reduces histopathology following axonal transection [29] or excitotoxic injury [8]. NGF infusion has also been shown to attenuate cognitive impairments but not motor impairments in rats after traumatic brain injury [26]. NGF levels are elevated following cerebral ischemia in the hippocampal CA1 region and decreased in other brain regions [12], and administration of exogenous NGF can reduce neuronal

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necrosis following cerebral ischemia [25,27,34]. It therefore appears that the production of neurotrophic factors is a feature of several types of CNS injury, and that neuronal injury may be attenuated by this response.

Neurotrophins have often been delivered to the CNS by cannulating the ventricular system [13,16,25,27] or intrathecal space [11,31], or by intraparenchymal infusion [26]. Although NGF appears to ameliorate the consequences of neuronal injury, the infusion process itself may damage the CNS or elicit inflammatory processes in the CNS, potentially resulting in upregulated production of substances such as neurotrophic factors [6,19,20]. Neuron survival after injury has been improved by infusion of artificial cerebrospinal fluid alone [10,25,27], as well as by implantation of atelocollagen pellets directly into the hippocampus [34]. This protective effect is analogous to findings that the placement of sham grafts into the striatum can induce behavioral recovery after MPTP-induced dopamine neuron lesions in primates, mimicking the beneficial effects of fetal tissue grafts [6,20]. It has been speculated that a mechanism of recovery following tissue grafting to the CNS may be elicitation of neurotrophin production in the host brain resulting from limited damage of the grafting procedure itself [6,20].

In the present study, we sought to determine whether central infusions of nerve growth factor (NGF) or artificial cerebrospinal fluid (aCSF) would ameliorate functional deficits in a well-characterized model of adult rabbit spinal cord ischemia [3,35]. Although NGF appears to limit hippocampal ischemic injury, the hippocampus is more sensitive to ischemic damage than any other brain regions and is therefore unrepresentative of broader neuronal populations. Thus, it is not clear whether NGF protection also extends to other CNS cell groups. Various neuronal populations exhibit specificity for different neurotrophic factors; for example, basal forebrain cholinergic neurons are primarily responsive to NGF, dopaminergic neurons to glial cell-line derived neurotrophic factor (GDNF), and motor neurons to CNTF, BDNF or GDNF (see e.g. Refs. [10,17,23,24,30–33]). In the spinal cord, low-affinity neurotrophin receptors have been detected on injured but not intact motoneurons of the spinal cord [14], suggesting that motor neuronal responses to injury may be regulated by growth factors. Further, sensory projections to the spinal cord express both low- and high-affinity neurotrophin receptors throughout life, and robustly extend new axons when provided with NGF after injury [28]. Finally, reactive astrocytes and microglia express neurotrophins after injury in the CNS, suggesting that more diverse and widespread effects of neurotrophins may act to influence spinal cord responses to injury [15,21].

To date, few studies that have examined the effects of NGF administration in models of CNS ischemia have also evaluated functional outcomes, despite several reports of NGF-induced neuroprotection. In the present experiment, we evaluated functional neurological outcome 18 h and 4

days after reversible spinal ischemia in rabbits. The intrathecal space was cannulated and infused with NGF or aCSF continuously for 3 days prior to induction of ischemia, and continuously for 4 days thereafter. Functional outcomes and NGF levels in the spinal cords were then assessed.

2. Materials and methods

2.1. Experimental subjects

Male New Zealand White rabbits (2–3 kg) were individually housed and provided with food and water ad libitum until the morning of surgery.

2.2. Infusion solutions

Infusion vehicle consisted of a phosphate-buffered artificial cerebrospinal fluid (aCSF) containing 150 mM NaCl, 1.8 mM CaCl_2 , 1.2 mM MgSO_4 , 2.0 mM K_2HPO_4 , and 10.0 mM glucose adjusted to pH 7.4. Recombinant NGF (300 g/ml in aCSF; generously supplied by Genentech) was administered to 21 animals, and 21 subjects received infusions of aCSF alone. Additional subjects [34] underwent spinal ischemia/reperfusion without intrathecal cannulation or infusions.

2.3. Placement of infusion tubing

Rabbits were anesthetized with inhaled halothane (5% induction, 2% maintenance by face mask). A #15 blade was used to incise the posterior pericervical skin layers, and muscle layers were bluntly dissected free from the region of the cervico-cranial junction to the level of the posterior spinal fascia. The fascia was then carefully incised with a #11 blade, creating a window through which a flexible Tygon catheter (outer diameter 0.03 inch) was inserted into the intrathecal space. A 15-cm length of tubing was gently advanced into the subarachnoid space, leaving the catheter to rest at the approximate T10 level of the spinal cord. Great care was taken to avoid direct damage to the spinal cord by halting insertion of the catheter if any resistance was encountered during the placement procedure. If resistance was encountered, the catheter was withdrawn for a distance of approximately 2 cm, then gently advanced again. Histological studies have shown that this procedure produces no tissue damage. Animals that sustained direct spinal cord damage from the insertion procedure were detected by observing locomotor function (ability to stand and walk normally) upon recovery from anesthesia; any animals with functional deficits were excluded from the study. Only subjects with normal locomotor function were included in subsequent studies of ischemia ($n=21$ NGF-treated, $n=21$ aCSF-treated, and $n=24$ lesioned/non-infused controls).

Upon completion of catheter insertion, the proximal end of the infusion tubing was connected to an Alzet Model 2ML2 mini-osmotic pump containing the experimental solution. The pump was placed subcutaneously in the infrascapular space of the animal and continuously delivered the experimental substance of interest at a rate of 71.4 $\mu\text{l/day}$.

2.4. Placement of aortic ligature device

Three days after placement of the intrathecal infusion catheter, experimental animals underwent a second surgical procedure for placement of the descending aorta ligature device, as previously described [36]. Animals were reanesthetized with halothane, and the abdominal aorta was exposed at the level of the renal arteries using a paramedial incision. Small-diameter plastic tubing (outer diameter 0.03 inch) was placed around the aorta just distal to the renal arteries. The ends of the tubing were threaded through a small plastic button and then through a plastic tube of larger diameter (outer diameter 0.125 inch), forming a snare ligature. The incision was closed around the large-diameter tubing so that the free ends of the tubing were accessible externally. The animals were allowed to recover for at least 2 h, and all displayed normal behavior prior to induction of spinal ischemia.

2.5. Induction of spinal cord ischemia

The animals were restrained and aortic occlusion was performed by pulling and clamping the small tubing around the aorta. Complete paraplegia was observed in all animals within 3 min of occlusion. Occlusion durations encompassing all grades of neurological outcome, from complete recovery to permanent paraplegia (15–50 min of occlusion), were selected (see Fig. 1). At the end of the ischemic period, the tubing was released to restore blood flow through the aorta. The tubing was removed, the abdominal wall closed with a suture that was placed during the surgery, and the skin closed with one or two wound clips. Animals were returned to their home cages and maintained for 4 days. Rabbits that died within this period were excluded to ensure that no animals with aortic thrombosis were included in the data analysis. All enzyme-linked immunosorbent assay (ELISA) samples were analyzed simultaneously; thus, CSF and pump samples from NGF-treated subjects were diluted to fall within anticipated NGF ranges of samples from vehicle-infused and intact subjects.

Neurological function was evaluated at 18 h and 4 days after ischemia/reperfusion by an observer blinded to the duration of ischemia and to the treatment group. Animals were classified by the presence or absence of paraplegia, as previously described [36]. This functional outcome model has been validated in several previous studies as a reliable means of assessing neuroprotection after ischemia. Ani-

mals were classified as 'paraplegic' if they showed no motor response to noxious stimuli in the hindlimbs and were completely incontinent. Rabbits were classified as 'not paraplegic' either if they were normal, or had any motor function of the hindlimbs, including motor function that was only modest in extent. Immediate postoperative continence of bladder function was required for animals to be classified in the 'not-paraplegic' group. In cases where the distinction between 'paraplegic' and 'not paraplegic' was uncertain, the animal was classified as paraplegic. Thus, the functional evaluation scale was conservatively biased.

Prior to sacrifice, animals were anesthetized with halothane, and 3-cc CSF fluid samples were withdrawn from the cisterna magna by transdermal C1–2 puncture. Pumps were removed from the backs and residual fluid volumes were measured to ensure adequate pump emptying during the experimental period. Animals were then killed using Beuthansia-D (Schering-Plough, Kenilworth, NJ), and lower thoracic spinal cords were extruded onto foil cooled on dry ice. The spinal cords were stored at -80°C . NGF levels were assayed by two-site ELISA in residual pump fluid, CSF, and lower thoracic spinal cord (T10 level), as previously described [33].

2.6. Data analysis

Neurological damage as a function of ischemic insult was analyzed using quantal dose–response analysis techniques described previously [32,37]. A computer was used to fit logistic (s-shaped) curves to the fraction of abnormal animals as a function of ischemic duration. The ischemic duration necessary to produce permanent paraplegia in 50% of a group of subjects was computed for each experimental group (the ET_{50} , for Effective Time). Pharmacological manipulations that improve neurological outcome increase ET_{50} , generating a shift of the dose–response curve to the right (see Fig. 1). The quantal bioassay allows the evaluation of dose–response curves spanning a wide degree of ischemic insult in an efficient manner and using a limited number of subjects, as previously described [37].

The ET_{50} s of the control, aCSF, and NGF groups were examined using one-way analysis of variance. A significant analysis of variance was followed with Tukey's test for multiple comparisons, with $P < 0.05$ considered significant. NGF ELISA values amongst groups were compared using analysis of variance, and post-hoc comparisons were made using Fisher's least square difference.

3. Results

3.1. Functional studies

Compared to non-infused control subjects, intrathecal

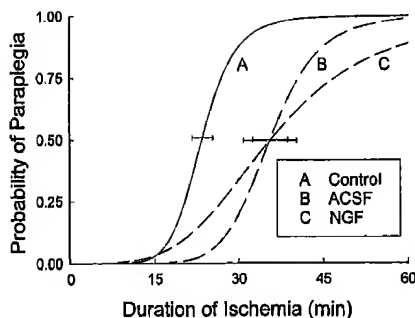


Fig. 1. Probability of paraplegia increases with increasing duration of spinal occlusion. For each group, the ischemia duration associated with a 50% probability of paraplegia (the ET_{50} , for Effective Time), and the S.E.M. are calculated. Intrathecal infusion of artificial cerebrospinal fluid (aCSF) or nerve growth factor (NGF) for 3 days prior to and 4 days following ischemia/reperfusion increased the duration of ischemia required to produce permanent paraplegia. NGF administration was no more effective than infusion of aCSF alone.

infusion of aCSF or NGF improved neurological outcome following reversible spinal ischemia and reperfusion (Fig. 1). At the 18-h evaluation, the $ET_{50} \pm S.E.M.$ were as follows: control non-infused, 25.7 ± 1.9 min; control aCSF-infused, 37.8 ± 3.0 min; NGF-infused, 33.05 ± 4.9 min ($P < 0.05$). Post-hoc comparison using Tukey's test at the 18-h evaluation indicated that the ET_{50} in the aCSF-infused group increased significantly compared to the non-infused lesion group; although the NGF group displayed a trend toward improved outcome, it was not significant. Four days after ischemia/reperfusion, significant improvement was observed in both the NGF-infused and aCSF-infused groups compared to the lesioned, non-infused group. The ET_{50} s were: control non-infused, 23.37 ± 1.8 min; control aCSF-infused, 35.5 ± 3.1 min; NGF-infused, 35.6 ± 4.7 min ($P < 0.05$).

ELISA measurement of NGF levels in freshly dissected spinal cords from the T10 region demonstrated significantly more NGF in infused subjects than in non-infused controls: 9.8 ± 3.8 ng NGF/g in NGF-infused subjects, 2.0 ± 0.4 ng NGF/g in aCSF-infused subjects and 0.4 ± 0.2 ng NGF/g in lesioned, non-infused animals ($P = 0.02$). Post hoc analysis indicated that all treatment groups differed significantly from one another with respect to NGF levels (Fig. 2). NGF ELISA measurements in the CSF of NGF-infused animals exceeded 600 ng/ μ l (upper limits of assay condition), compared to undetectable levels in aCSF-infused subjects and non-infused subjects. Similarly, NGF levels exceeded 600 ng/ μ l in residual pump fluid from NGF-infused subjects, and was undetectable in residual pump fluid withdrawn from aCSF-filled pumps.

NGF ELISA Results

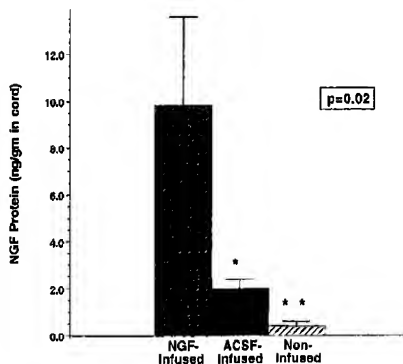


Fig. 2. NGF levels in spinal cord 4 days after induction of ischemia, as determined by ELISA.

4. Discussion

In the present experiment, cannulation of the intrathecal space and continuous infusion of either artificial CSF or human NGF resulted in significant neuroprotection after transient spinal cord ischemia. Artificial CSF alone or NGF in artificial CSF were administered continuously, beginning 3 days prior to induction of ischemia and continuing until sacrifice 4 days later. Both groups exhibited significantly improved functional outcomes compared to animals that were subjected to ischemia and did not receive intrathecal instrumentation. Interestingly, levels of NGF were elevated in the ischemic spinal cord of both groups of cannulated animals, and were not elevated in the cords of animals that received lesions without instrumentation. Thus, the process of intrathecal instrumentation itself appears to have elevated neurotrophin levels in the ischemic spinal cord to levels sufficient to elicit neurotrophin production, a finding that correlated with enhanced functional recovery. Although NGF levels were approximately 5-fold greater in the cords of NGF-infused subjects, the extent of functional recovery in these animals was not greater than that observed in lesioned, aCSF-infused subjects. These findings suggest that modest, physiological rises in neurotrophin levels may be sufficient to improve outcomes after spinal cord ischemia.

These findings are consistent with previous studies demonstrating that infusion of artificial CSF improves outcome following injury [25,27]. The mechanism by which neuroprotection occurs in these studies is not known. At least two possibilities must be considered: the process of cannulating the intrathecal space may mildly

traumatize the spinal cord and the spinal nerve roots, potentially leading to secretion of inflammatory molecules, cytokines, or trophic factors that promote neuroprotection from the subsequent ischemic deficit. Following CNS trauma, molecules possessing neurotrophic activity are detectable in the CNS [19]. Previous studies by Kordower [6] and Bankiewicz [20] have reported partial recovery of motor function in hemiparkinsonian monkeys resulting simply from lesion placement in the brain. In these studies, it was hypothesized that minor trauma in the brain induced production of neurotrophic factors and neuritic sprouting that could have mediated partial functional recovery. The present findings support natural growth factor elevation after trauma in the CNS as a potential mechanism of generating functional recovery. Nervous system injury also stimulates cytokine release that may be neuroprotective [4]. Thus, the insertion of a catheter into the thecal space 3 days before injury in the present experiment could have primed the system to express neuroprotective molecules that subsequently reduced the extent of ischemic deficit. This finding was not observed, however, in a previous study in which the intrathecal space was cannulated and drugs were administered as a single injection rather than as a sustained infusion [2]. Thus, the presence of an ongoing stimulus may be required to elicit the release of neuroprotective substances. The rate of fluid infusion in this model is sufficiently low (71.4 μ l/day) compared to the volume and rate of CSF formation in rabbits that fluid flow from the catheter was unlikely in itself to have produced damage; rather, an irritative effect of the tubing would appear to be a more likely explanation for the observed effect. Rabbit serum (0.1%) was added to the infusate solution in both NGF-treated and control-infused animals, and it is possible that it contained a neuroprotective substance. In vitro studies indicate improved cell viability in the presence of serum. However, a number of previous in vivo studies have failed to yield evidence of morphological neuronal protection after infusions of artificial CSF containing serum into the cerebral lateral ventricles, suggesting that serum does not mediate the protective effect [9,13,16,30].

An investigation of structural (morphological) changes underlying the observed functional effect is necessary to gain further insight into mechanisms underlying the present findings. The increased functional recovery with elevation in neurotrophic factor levels suggests that examination of neuronal number and lesion size would be informative and will be the subject of future studies.

This study suggests that endogenous mechanisms may be capable of providing significant neural protection after CNS ischemia or injury if an adequate stimulus is provided for the activation of these mechanisms. Whether such a stimulus must be provided before the neural insult, as was the case in the present study, or can be provided after the onset of injury, remains to be determined. A fruitful strategy for enhancing neural recovery after CNS injury in

general will be the search for substances that can stimulate the activation of endogenous repair mechanisms, such as trophic factors, to mimic findings of the present study.

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A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection

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Article abstract—*Objective:* To evaluate the safety and efficacy of recombinant human nerve growth factor (rhNGF) in HIV-associated sensory neuropathy (SN) within a multicenter, placebo-controlled, randomized trial (ACTG 291). *Background:* SN affects 30% of individuals with AIDS, is worsened by neurotoxic antiretrovirals, and its treatment is often ineffective. NGF is trophic for small sensory neurons and stimulates the regeneration of damaged nerve fibers. *Methods:* A total of 270 patients with HIV-associated SN were randomized to receive placebo, 0.1 µg/kg rhNGF, or 0.3 µg/kg rhNGF by double-blinded subcutaneous injection twice weekly for 18 weeks. The primary outcome was change in self-reported neuropathic pain intensity (Gracely Pain Scale). Secondary outcomes included an assessment of global improvement in neuropathy by patients and investigators, neurologic examination, use of prescription analgesics, and quantitative sensory testing. In a subset, epidermal nerve fiber densities were determined in punch skin biopsies. *Results:* Both doses of NGF produced significant improvements in average and maximum daily pain compared with placebo. Positive treatment effects were also observed for global pain assessments ($p = 0.001$) and for pin sensitivity ($p = 0.019$). No treatment differences were found with respect to mood, analgesic use, or epidermal nerve fiber densities. Injection site pain was the most frequent adverse event, and resulted in unblinding in 39% of subjects. Severe transient myalgic pain occurred in eight patients, usually from accidental overdosing. There were no changes in HIV RNA levels or other laboratory indices. *Conclusions:* We found a positive effect of recombinant human nerve growth factor on neuropathic pain and pin sensitivity in HIV-associated sensory neuropathy. rhNGF was safe and well tolerated, but injection site pain was frequent. **Key words:** Nerve growth factor—HIV-associated sensory neuropathy—Pain—Analgesic—Dideoxynucleoside.

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Patients with HIV infection frequently develop sensory neuropathy (SN), which usually presents with pain and paresthesias in the feet.^{1,2} The annual incidence of HIV-associated sensory neuropathy (HIV-SN) is 2%.³ Specific dideoxynucleoside antiretroviral agents can trigger or exacerbate this neuropathy; thus, HIV-SN can limit the continued use of these agents.^{4,5} To date, the only treatments available for HIV-SN have been symptomatic, including pain-modifying agents, such as antidepressants, anticonvulsants, or, in severe cases, narcotic analgesics.

Controlled trials in HIV-associated SN have failed to demonstrate significant effects on neuropathic symptoms or pain levels.^{6,7}

The pathology of HIV-SN is a length-dependent degeneration of peripheral nerve fibers, with prominent involvement of small myelinated and unmyelinated fibers subserving nociception.⁸ Whereas the pathogenesis remains unknown, this damage to small sensory nerve fibers presents a potential target for nerve growth factor (NGF), which is neurotrophic for nociceptive fibers.^{9–11} NGF is critical within

*Other participants in the study are listed in the Appendix on page 1087.

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both the developing and the mature nervous systems, and is produced in the damaged peripheral nerve to stimulate collateral sprouting.¹² Experimentally, recombinant human NGF (rhNGF) improved diabetic and chemotherapy-induced neuropathies,¹³ and had positive effects on nerve fiber function in a Phase 2 trial for diabetic neuropathy,¹⁴ although a recently analyzed Phase 3 trial did not confirm this (A. Vinik, personal communication, May 1999). Consequently, in 1996 the AIDS Clinical Trials Group began a controlled clinical trial of rhNGF in patients with HIV-SN.

Methods. Subject selection. Adults with HIV-associated SN, confirmed by a neurologist using criteria developed by the American Academy of Neurology,¹⁵ were included. Physiologic abnormalities on nerve conduction testing or quantitative sensory testing (QST) were not incorporated as inclusion criteria. At baseline, patients were required to have neuropathic pain of at least "mild or moderate intensity" with no other identifiable cause of peripheral neuropathy such as diabetes mellitus, vitamin B₁₂ deficiency, or previous exposure to neurotoxic substances. Subjects with cognitive impairment or other conditions that might have prevented the accurate completion of self-rating pain scores were excluded. Subjects were stratified by use of didanosine (ddI), zalcitabine (ddC), or stavudine (d4T) as follows: current use, use discontinued between 8 and 26 weeks before randomization, use discontinued at least 26 weeks before randomization, or never used. Patients were excluded from entry if they had discontinued dideoxynucleosides within 8 weeks of randomization. Changes in dideoxynucleoside use during the study were permitted, but occurred in only 10 patients.

Study design. The double-blind phase of this study included an 18-week drug treatment phase followed by a 4-week washout period. Beginning in July 1996, subjects were enrolled at 17 sites, and randomly assigned to one of three arms in an equal fashion as follows: 0.1 µg/kg rhNGF self-administered subcutaneously twice weekly, 0.3 µg/kg rhNGF self-administered subcutaneously twice weekly, or rhNGF placebo self-administered subcutaneously twice weekly. Randomization was through the use of computer-generated permuted blocks with one stratification factor and balanced treatments within each institution. The centralized data management organization generated the randomization codes (L.M.), which were transmitted to site pharmacists, and allocation was completely concealed from the site personnel except for "emergency" allocation information maintained with each site pharmacist. Study visits were required every 2 weeks to check on injection site symptoms and to review the accuracy of the pain logs. Neurologic examinations were performed at initial screening and at weeks 6, 12, 18, and 22. Blinding questionnaires were administered at weeks 6, 12, 18, and 22. The questionnaire asked, "Based on your experience in the study, do you think that you are receiving the study treatment rhNGF or the placebo?" Reasons for answers were elicited, e.g., response or treatment-related side effects. The results from week 18 were utilized in the analysis of unblinding. Laboratory studies were performed at baseline and at weeks 4 and 18. After completion of the double-blind phase, subjects were permitted to enroll in a

Table 1 Gracely Pain Scale verbal descriptors of pain intensity and assigned log units used in analysis^{16,17}

Descriptor	Pain intensity	Log unit
A	Nothing	0
B	Faint	0.36
C	Very weak	0.40
D	Weak	0.45
E	Very mild	0.59
F	Mild	0.74
G	Moderate	1.09
H	Barely strong	1.10
I	Slightly intense	1.33
J	Strong	1.36
K	Intense	1.54
L	Very intense	1.64
M	Extremely intense	1.77

48-week open-label phase with active treatment. The results from this component will be reported separately. Informed consent, approved by individual institutional review boards, was obtained. A Safety Monitoring Committee, coordinated through the Division of AIDS, National Institute of Allergy and Infectious Diseases, received monthly reports on adverse events and laboratory toxicities.

Primary and secondary outcome measures. The Gracely Pain Scale was used to assess the intensity of neuropathic pain (table 1). Subjects selected verbal descriptors to match their neuropathic pain from a 13-item scale developed and validated in earlier studies.^{7,16,17} Subjects were instructed to record both average and peak/maximum neuropathic pain in a diary on a daily basis. The verbal descriptors were converted into log units using previously published values,^{7,16} and a weekly average was derived for statistical analyses. The primary efficacy endpoint was the change in self-reported pain intensity (average daily pain) measured using this scale, from baseline to week 18. Secondary endpoints included the change in peak/maximum daily pain intensity and global assessments of neuropathic pain by subjects and examining neurologists. Neurologic examination findings were assessed using a modification of the Neuropathy Impairment Score for Lower Limb.¹⁸ The pin sensation score was derived from the neurologist's examination as follows: normal pin sensation or distal hyperalgesia (scored as 1); diminished sharp sensation (scored as 2); and inability to sense sharp (scored as 3). The change in pin sensitivity was determined by subtracting the baseline score from the week 18 score. We measured vibratory and cooling detection thresholds using the Computer Assisted Sensory Evaluator (CASE-IV) (Stillwater, MN) system to quantify vibratory and cooling thresholds in fingers and toes. The 4-2-1 algorithm was used¹⁹ and two separate tests were run at each time point with the scores averaged. Use of prescription analgesics and scores of mood (Profile of Mood States [POMS]) were also recorded. As part of the monitoring of adverse events, we assayed plasma HIV RNA levels at baseline and weeks 4 and 18 using the Roche Amplicor kit (Indianapolis, IN).²⁰ In a subset of 60 subjects, we determined epidural

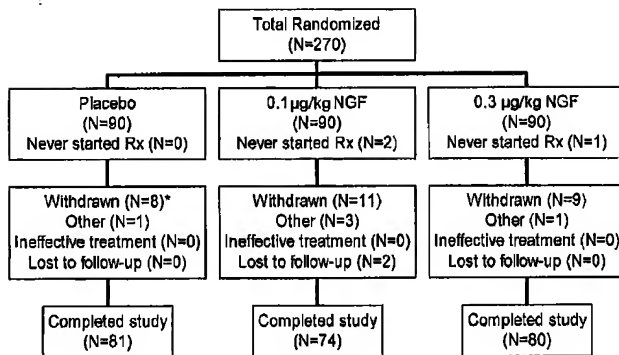


Figure 1. Trial profile: disposition of subjects. *Includes one death. rhNGF = nerve growth factor.

nerve fiber densities using punch skin biopsies at baseline and week 18. The microscopist was blinded to treatment assignment and to the order of specimens, and used previously published quantitation techniques.^{20,21}

Statistical analyses. The primary efficacy analysis involved comparisons of changes in average daily pain intensity from baseline to week 18 among the three treatment groups, stratified by patterns of baseline dideoxynucleoside use (current use, recent use, remote use, or nonuse), and was adjusted by baseline daily average pain levels. A last observation carried forward (LOCF) approach was used to impute missing data pertaining to weeks 1 through 18. Similar analyses based only on existing data were also performed. Results were similar in both analyses. Thus, there was no evidence that patient dropout was related to the study outcome. All reported results are from the LOCF analysis. The resulting statistical model was a two-way analysis of covariance (ANCOVA). In cases where a statistically significant overall treatment effect was observed, post hoc, pairwise comparisons between low-dose rhNGF and placebo and high-dose rhNGF and placebo were carried out. Significance levels were adjusted for the multiple comparisons by the Dunnett test.²² Differences among treatment groups with respect to non-normally distributed measures (e.g., QST) were tested using the Kruskal-Wallis test, whereas pairwise differences were compared by the Wilcoxon's rank sum test, at a Bonferroni-adjusted significance level. Finally, comparisons involving unordered categorical variables were performed with Fisher's exact test. Trends in ordered categorical variables were tested with the Mantel-Haenszel χ^2 test adjusted for dideoxynucleoside use. All statistical tests were two-tailed, and 95% confidence intervals are provided for the primary endpoints.

Results. *Baseline characteristics of study subjects.* A total of 270 subjects were enrolled into the double-blind phase of the trial at 17 sites (figure 1). Table 2 provides demographic characteristics at baseline, showing that the treatment groups were well balanced with respect to age, sex, baseline plasma HIV RNA levels and CD4⁺ lymphocyte count, history of injection drug use (8.9% overall), use of opioid analgesics, and severity of neuropathic pain. The treatment groups were also well balanced with respect to baseline abnormalities on QST and use of dideoxynucleoside antiretrovirals. Only 38% of subjects were using opi-

oid analgesics at entry, and most of the participants were using either nonopioid analgesics or adjunctive pain-modifying agents. At baseline, among all subjects, the mean Gracely value for average daily pain was 1.00 log units (95% CI 0.96 to 1.04), corresponding approximately to "moderate" neuropathic pain. The median Cooling Detection Threshold for the foot was at the 96th percentile and the distribution of QST results was comparable among the groups. Overall, 44% of subjects had values within a normal range, i.e., below the 95th percentile.²³ Fifty-five percent of placebo recipients were abnormal, compared with 58% of the 0.1 µg/kg group and 52% of the 0.3 µg/kg group (χ^2 0.561, p = 0.755). Epidermal nerve fiber densities in 60 subjects were 18.2 (16.68 to 19.72) fibers/mm at the thigh and 8.34 (7.06 to 9.69) fibers/mm at the distal calf, compared with control values of 21.1 and 13.8, respectively.²⁴ Forty-three percent had values at the distal part of the leg below the tenth percentile cutoff.

Study experience. A total of 226 subjects (85%) completed the treatment period, 44 (15%) discontinued prematurely, and 3 (1%) were randomized but never treated. Table 3 details the treatment experience among the three treatment groups. Only two subjects (both in the 0.1 µg/kg group) discontinued because of protocol-related toxicity.

Effects on neuropathic pain intensity. A significant difference among treatment groups was noted for changes in average and maximum pain intensity from baseline to week 18 favoring rhNGF. Pairwise p values for comparisons between placebo and higher dose rhNGF for average and maximum pain were 0.05 and <0.01, and for lower dose rhNGF 0.04 and <0.01. No significant dose effect was observed. Figure 2A illustrates the improvement in average neuropathic pain from baseline to week 18, and figure 2B shows the improvements over the course of the study.

The mean adjusted improvements (adjusted for baseline average daily pain intensity) for placebo, 0.1 µg/kg rhNGF, and 0.3 µg/kg rhNGF were 0.06 (0.01 worsening to 0.14 improvement), 0.18 (0.10 to 0.25), and 0.21 log units (0.14 to 0.29). Subjects with greater pain at baseline tended to experience greater improvement than those with less intense neuropathic pain. There was no differential effect of treatment based on the baseline stratification for use of dideoxynucleoside antiretrovirals, and no effect on study outcomes among the 10 subjects who changed dideoxynucleoside use during the study.

Table 2 Baseline characteristics of study subjects

Characteristic	Placebo, n = 90	rhNGF 0.1 µg/kg, n = 90	rhNGF 0.3 µg/kg, n = 90	Overall, n = 270
Age, y, mean (SD)	43.6 (8.8)	44.5 (8.8)	43.7 (8.6)	44.0 (8.7)
Sex, % male	97.8	97.8	96.7	97.4
Race, % white (non-Hispanic)	87.8	88.9	87.8	88.1
IV drug history, %	6.7	10.0	10.0	8.9
Concurrent use of narcotic analgesics,* %	37	30	40	38
CD4 count (cells/mm ³)				
Mean (SD)	205.9 (146.3)	209.3 (144.6)	223.1 (196.1)	212.9 (164.0)
Median	181.5	186	168	175
Average pain† (log units)				
Mean (SD)	0.98 (0.35)	1.01 (0.34)	1.00 (0.30)	1.00 (0.33)
Median	1.0	1.0	1.0	1.0
Quantitative sensory testing, % abnormal (≥95 percentile)				
Foot vibration	58	68	58	61
Foot cooling	55	58	52	55

* Only analgesic regimes containing opioids were classified as *narcotic* analgesics.

† Gracely Pain Scale. Daily pain scores averaged over 1 to 2 weeks and converted to logarithmic units using previously published values^{16,17} and as noted in table 1.

rhNGF = recombinant human nerve growth factor.

Analysis of the global assessment of pain showed that significantly more subjects treated with rhNGF reported improvement in neuropathy at weeks 12 or 18, or were rated as improved by examining neurologists. Fifty-one percent and 37% of patients randomized in the higher and lower NGF arms rated their neuropathic pain as "improved" or "much improved" at week 12, compared to 23% among the placebo patients (overall test $p < 0.001$; lower rhNGF versus placebo 0.21; higher rhNGF versus placebo <0.001 , based on Mantel Haenszel test). At week 18, 17%

of placebo recipients rated their neuropathy as "improved" or "much improved" compared to 33% of lower dose and 36% of higher dose patients (overall test $p = 0.048$; the Bonferroni-adjusted p values for lower dose versus placebo $p = 0.132$; and for higher dose versus placebo $p = 0.024$). These results, and those of the examining neurologist ratings, are displayed in figure 3.

Effects on neurologic examination. We found a significant improvement in pin sensitivity determined by blinded neurologic examination (figure 4) for the combined NGF

Table 3 Subject experience during the double-blind study period

Characteristic	Placebo n = 90	rhNGF 0.1 µg/kg n = 88	rhNGF 0.3 µg/kg n = 89	Overall n = 267
Treatment status*				
Completed, n (%)	80	70 (78.4)	76 (85.4)	226 (84.6)
Discontinued, n (%)	10 (11.1)	18 (20.5)	13 (14.6)	41 (15.4)
Reasons for discontinuations				
Total no. discontinuations	10	18	13	41
Death	1†	0	0	1
Toxicity per protocol	0	2	0	2
Toxicity nonprotocol	2	5	8	15
Subject request	5	9	3	17
Other‡	2	2	2	6
Open label enrollment	73	61	66	200

* Three subjects never started treatment.

† Suicide.

‡ Two subjects were discontinued because of use of experimental agent or other drug(s) with known antiviral activity, other than study drug(s), and seven discontinued for miscellaneous reasons.

rhNGF = recombinant human nerve growth factor.

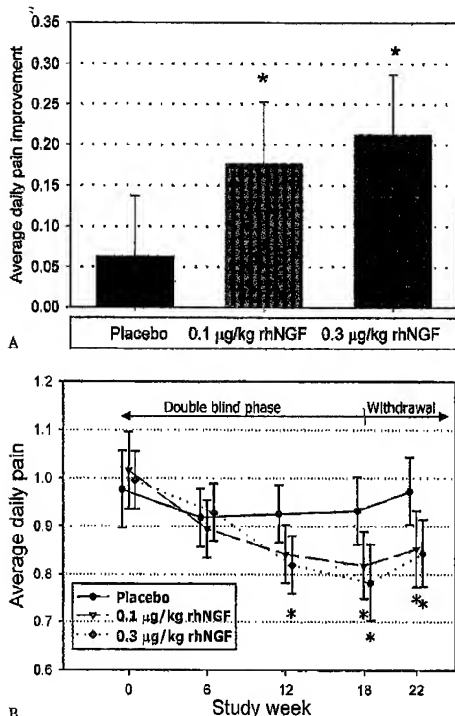


Figure 2. (A) Changes in average pain intensity. Plotted are adjusted means \pm 2 SEM. Asterisks indicate statistically significant differences compared with placebo. rhNGF = recombinant human nerve growth factor. (B) Changes in average pain intensity over the 22 weeks of the study. Adjusted mean \pm 2 SEM are plotted. Asterisks indicate statistically significant differences compared with placebo.

treatment arms and a small deterioration in the placebo group ($p = 0.019$). The Bonferroni-adjusted pairwise comparisons corresponding to comparisons of placebo and higher dose rhNGF were $p = 0.078$ and for placebo and lower dose rhNGF were 0.015. We found no changes in other components of the neurologic examination, including vibratory sensibility, muscle strength, or deep tendon reflexes.

Effects on QST, analgesic use, and depressive symptoms. Overall, no significant treatment effects on either cooling or vibratory quantitative sensory thresholds were noted. A subgroup analysis of subjects who had abnormal cooling thresholds at baseline (≥ 95 th percentile) showed that sensory thresholds were closer to normal at week 18 for higher dose rhNGF recipients compared with placebo (Bonferroni-adjusted, $p = 0.016$ "just noticeable differences" and $p = 0.012$ for percentile scores; see figure 5, A and B). The corresponding p values for the lower-dose rhNGF group versus placebo were 0.392 and 0.034. The changes from baseline to week 18 were not statistically significant.

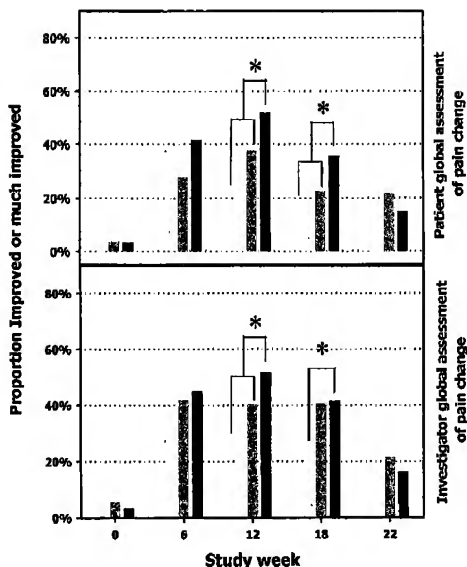


Figure 3. Investigator and patient "global" assessments of change in pain from baseline. The baseline assessment (week 0) change in pain refers to changes during the 2-week screening period before treatment. Asterisks mark statistically significant differences from placebo. Light gray line, placebo; medium gray line, 0.1 µg/kg recombinant human nerve growth factor (rhNGF); black line, 0.3 µg/kg rhNGF.

A blinded review of prescription analgesic use at baseline and week 18 showed no significant decrease in the amount or class of analgesic use reported by study participants. Additionally, no effect on self-reported mood or psychological symptoms was found, using POMS.

Effects on epidermal nerve fiber density. Punch skin biopsies were performed in 60 subjects at baseline and

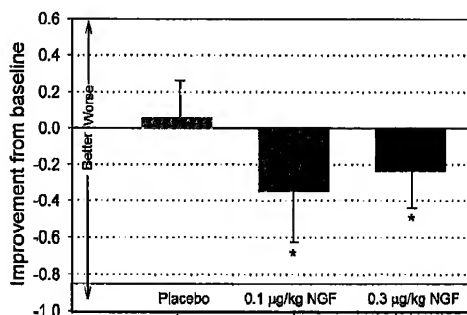


Figure 4. Changes in pinprick sensation from the standardized neurologic examination. Asterisks indicate statistically significant differences from placebo. rhNGF = recombinant human nerve growth factor.

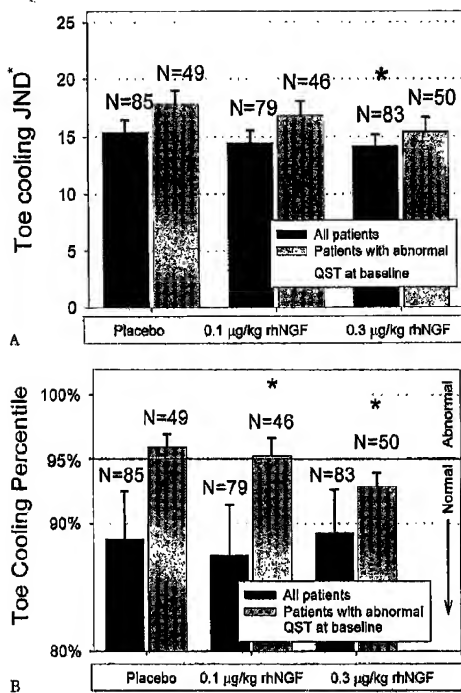


Figure 5. (A) Analysis of toe cooling thresholds at week 18 from the CASE IV quantitative sensory testing (QST) for all patients (black bars) and only those subjects with abnormal QST (>95 percentile). Plotted are mean just noticeable difference (JND) values \pm 2 SEM. Asterisks indicate statistically significant differences from placebo. rhNGF = recombinant human nerve growth factor. (B) Analysis of toe cooling thresholds at week 18. Plotted values are mean percentile scores \pm 2 SEM. Asterisks indicate statistically significant differences from placebo.

week 18: 19 in the placebo group, 21 in the low-dose NGF, and 20 in the high-dose subjects. The reproducibility of the technique was assessed from intrasubject correlations between baseline and week 18 densities. The overall estimate of the correlation was 81% in the lower leg and 77% in the upper thigh (p value < 0.001 in both cases).²⁴ No significant treatment effect was observed over the 18 weeks.

Adverse events. rhNGF was well tolerated in the study subjects, and only two subjects discontinued treatment because of protocol-related toxicity (see table 3). One death occurred during the study period: a suicide in a subject receiving placebo. A total of 101 subjects experienced adverse events during the first 22 weeks of study (a total of 126 events were reported): 25 subjects (29 total events) in the placebo group, 22 (31 events) on the lower dose NGF, and 54 (66 events) on the higher dose NGF. The most frequent adverse events were injection site pain or myalgias, which were reported in 12 placebo recipients (15 total occurrences), 17 (25 occurrences) in lower dose rhNGF, and 36 (48 occurrences) in higher dose rhNGF subjects. The local injection site pain was dose-related, and usually lasted 10 to 20 days after each injection. Severe myalgias occurred in eight subjects and was usually attributable to "accidental" overdosage with rhNGF because of dilution errors. No significant hematology or chemistry toxicities were observed, and there was no significant treatment effect on plasma HIV RNA levels determined by Roche Amplicor assay¹⁹ between baseline, week 4, and week 18.

Impact of unblinding during the study. The impact of injection site pain on unblinding was assessed with a questionnaire at weeks 6, 12, 18, and 22. Subjects' responses were then categorized by whether unblinding was response-related or based on treatment side effects. From 244 subjects who completed the blinding questionnaire, 96 (39%) guessed treatment assignment because of injection-related symptoms. Table 4 demonstrates the mean changes in average daily pain by blinding status. A subgroup analysis was performed to attempt to eliminate the effects of unblinding from injection site pain or other adverse events. We combined participants who remained blinded throughout the study and those who were un-

Table 4 The effects of unblinding on changes in neuropathic pain: Difference from baseline to week 18

Subject subgroup	Treatment assignment		
	Placebo	0.1 µg/kg rhNGF	0.3 µg/kg rhNGF
Combined group (subjects who remained blinded and those who guessed by treatment response)*	n = 74	n = 44	n = 39
Average Gracely pain improvement	0.05 (0.04)	0.14 (0.05)	0.20 ^a (0.05)
Maximum Gracely pain improvement	0.08 (0.04)	0.18 (0.05)	0.27 ^b (0.05)
Subjects unblinded due to adverse events	n = 10	n = 35	n = 42
Average Gracely pain improvement	0.19 (0.09)	0.24 (0.06)	0.24 (0.05)
Maximum Gracely pain improvement	0.29 (0.10)	0.29 (0.06)	0.28 (0.05)

All means have been adjusted for baseline pain. Statistically significant differences from placebo, ^a p = 0.039 and ^b p = 0.009. Statistical comparisons were based on the Dunnett's post-hoc test. Numbers in parentheses denote standard errors of the adjusted means.

* The combined group includes subjects who remained blinded throughout the study as well as those who correctly guessed receiving NGF due to improvement.

rhNGF = recombinant human nerve growth factor.

blinded because of treatment response (and *not* from injection site pain). Even though this "combined" subgroup was smaller than the total study population, significant improvements in both average and maximum pain were observed for the higher rhNGF dose. The group that was unblinded by adverse events showed no significant treatment effect.

NGF serum levels and antibodies. Levels of antibodies to rhNGF and rhNGF levels in plasma were determined at week 18 in 60 randomly selected samples using published techniques.¹⁴ All antibody titers were negative, and the random plasma rhNGF levels were below the limits of detection (12 pg/mL).

Discussion. This study represents the first use of rhNGF in HIV-associated SN. We tested the administration of rhNGF for 18 weeks in 270 patients, and found a significant reduction in neuropathic pain, as well as improved pin sensibility on neurologic examination. NGF was well tolerated, with few serious adverse events. Injection site pain or hyperalgesia, however, was frequent, dose-related, and usually lasted 10 to 20 days after each injection. Only two subjects discontinued because of protocol-related toxicities. Despite its frequency, injection site pain did not frequently prompt study discontinuation; however, about a third of subjects were potentially unblinded by this stereotypic adverse effect. Diffuse myalgias or "neuromyalgia," which had been reported in other studies of rhNGF,²⁶⁻²⁷ occurred rarely, and usually as a consequence of accidental overdosage. The mechanism of the myalgias remains uncertain, but was usually self-limited with no lasting effect on muscle strength. It has not occurred in the open-label continuation study with a different formulation of rhNGF that requires no dilution.

The treatment-related changes in the average daily Gracely pain scale corresponded to net improvements of 0.12 and 0.15 log units after correcting for placebo effect. The differences became significant by 12 weeks of treatment, but were independent of dose. In other pain studies changes of this magnitude have been considered clinically meaningful, corresponding to an improvement of about one grade on the Gracely scale for pain scores in the "moderate range" and about two grades for pain scores in the "severe" range.¹⁶ The Gracely scale is based on verbal descriptions of pain, some of which are very similar, e.g., "slightly intense" and "strong." Nonetheless, studies have demonstrated its reliability for measuring changes in neuropathic pain.¹⁷ Furthermore, the results on the Gracely are consonant with the patient and investigator global ratings. When we accounted for unblinding from injection site symptoms (as opposed to correct guessing of treatment because of response), the treatment-related differences were attenuated, losing significance. However, in many patients with pain who remained blinded, significant changes were noted for both average and maximum daily pain. We excluded individuals with cognitive

impairment, who might have had difficulty completing the daily pain logs.

The QST with the CASE IV device showed no changes, in contrast to the Phase II diabetic neuropathy trial in which rhNGF had an effect on heat pain threshold.¹⁴ Our sensory testing protocol included only cooling and vibratory thresholds, which are sensitive to small myelinated A delta and larger myelinated A beta fibers, respectively. An effect on C fibers, which subserve heat pain,^{28,29} and include NGF-responsive fibers, might therefore have been missed.^{18,28} In addition, we did not mandate abnormal QST for entry, and overall, 44% of subjects had testing within a normal range. Restricting the analysis to those with abnormal sensory testing at baseline (i.e., scores above the 95th percentile) demonstrated improved cooling thresholds, suggesting an effect of higher dose rhNGF. This must be interpreted cautiously, however, as no significant effect was seen for the lower dose of rhNGF, and this was a subgroup analysis involving one component of a secondary outcome variable.

The use of punch skin biopsies for the diagnosis and evaluation of sensory neuropathies was first widely used by Bolton and Dyck^{30,31} and has been subsequently refined by other groups.³²⁻³⁶ The availability of the sensitive panaxonal marker PGP9.5, combined with the validation of simplified quantitation techniques,³⁶ has facilitated the study of idiopathic small fiber SN³⁰ and HIV-associated SN.^{21,37} This trial represents the first use of epidermal nerve fiber densities as a therapeutic outcome measure. The reproducibility of the technique was good; taking all samples, week 18 epidermal nerve fiber densities were closely correlated with baseline densities. However, only a minority of subjects (43%) had abnormal epidermal nerve fiber densities at the distal leg site at baseline, and morphologic changes were in general less dramatic than were observed in idiopathic small fiber SN.³⁰ This suggests that the pathologic severity of neuropathy in these subjects was relatively mild. Whereas no significant changes in epidermal nerve fiber density were observed after 18 weeks of treatment, the open label study is continuing and information will be available after 70 weeks of rhNGF treatment. It is likely that regeneration or collateral sprouting of nerve fibers in the epidermis may take substantially longer than the 18 weeks of this study. For example, experimental studies focusing on the time course of intraepidermal nerve fiber regeneration within mouse foot pads following sciatic nerve injury have emphasized that reinnervation of the epidermis is often delayed even though reinnervation of dermal structures may occur promptly (e.g., sweat glands are fully reinnervated 39 days after crush injury).³⁸ In a patient we studied with diabetic truncal neuropathy, epidermal reinnervation was detected 2 years after the original injury.³⁹ The reasons for delayed epidermal reinnervation after nerve injury are unknown, but it is not related directly to the distances from nerve injury to epidermal target. One

hypothesis is that there is a defect in axon guidance due to loss of Schwann cell bands.^{3a}

It is frequently difficult to differentiate clinically HIV-associated SN from the toxic effects of dideoxynucleoside antiretroviral agents, particularly in individuals with advanced HIV infection.⁵ The mechanisms of dideoxynucleoside-induced peripheral nerve damage may include the inhibition of mitochondrial DNA polymerase.^{40,41} We found no treatment differences after stratification by exposure to dideoxynucleoside analogues, suggesting that toxic neuropathies may respond to rhNGF in a similar manner to HIV-associated neuropathy not associated with these toxic agents. This observation has clinical relevance because the development of SN often limits the continuation of specific antiretrovirals. rhNGF might be useful in this situation to either prevent the development of neuropathy or attenuate its progression in patients receiving dideoxynucleoside antiretrovirals.

Limitations of this study include the apparent unblinding of a proportion of subjects because of the injection site symptoms. In future studies, we may focus on physiologic outcomes or utilize an active placebo or some method of masking injection site pain to counter this. Most of the subjects did not have severe neuropathy based on baseline levels of self-reported pain, results of QST, and epidermal nerve fiber densities. The inclusion of relatively mild neuropathy cases might have "diluted" the results, as we observed the more robust treatment effects in those with more severe neuropathic pain. Finally, the dosing frequency in our study was twice weekly in comparison to the three times weekly dosing used in the recent diabetic neuropathy trial (personal communication, A. Vinik, 1999). This frequency was selected empirically, and as random plasma samples showed undetectable levels of rhNGF, the systemic levels must have been low with this dose and dosing frequency. Treatment effects might have been more pronounced with a higher dosing frequency.

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Appendix

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The members of the *National Institute of Neurological Disorders and Stroke* Performance and Safety monitoring board were as follows: B. Jubelt (chair), State University of New York Health Science Center, Syracuse; B. Barton, Medical Research Institute, Baltimore; J. Noseworthy, Mayo Clinic, Rochester, MN; L. Sharer, UMDNJ Medical School, Newark; and A. Kerza-Kwiatkowski, National Institute of Neurological Disorders and Stroke, Bethesda, MD.

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Disclosure

Dr. Katzenstein has received honoraria from Genentech in association with meetings discussing projects related to HIV neuropathy within the last 5 years.

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